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NON-ALCOHOLIC FATTY LIVER DISEASE

Studies on pathogenesis and diagnosis

Elina Isokuortti

ACADEMIC DISSERTATION

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To my family

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a disease spectrum in which excess fat accumulates in the liver. NAFLD often coexists with obesity, metabolic syndrome and type 2 diabetes ('Metabolic NAFLD') and is characterised by insulin resistance. NAFLD may also be due to common gene variants in *PNPLA3* at rs738409 and *TM6SF2* at rs58542926. It is unclear whether these forms of 'Genetic NAFLD' are related to insulin resistance. Liver fat content may be assessed using liver histology, imaging tools or biomarkers. This thesis was undertaken to better understand the pathogenesis of NAFLD and to improve currently available diagnostic tools.

Subcutaneous (SC) adipocyte hypertrophy is associated with insulin resistance, but it is unknown whether SC adipocyte size is independently associated with liver fat content. In study I, mean adipocyte size was determined from a SC adipose tissue obtained from 119 non-diabetic subjects, whose liver fat content was measured using proton magnetic resonance spectroscopy (^{1}H-MRS). SC adipocyte size significantly associated with liver fat content independent of age, gender, measures of body composition and *PNPLA3* genotype (R^2=54%, p<0.0001).

In study II, a systematic review was conducted to investigate whether 'PNPLA3 NAFLD' and 'TM6SF2 NAFLD' are associated with insulin resistance. In 12 of 14 studies, the carriers of the PNPLA3 I148M variant had higher liver fat content than the non-carriers without an increase in insulin resistance, while in 5 of 7 studies, the carriers of the TM6SF2 E167K variant had higher liver fat content than the non-carriers without an increase in insulin resistance. A systematic review was also performed to compare how normal liver fat content is defined by liver histology and currently available imaging tools: ^{1}H-MRS, magnetic resonance imaging, computed tomography and ultrasound. The definitions of normal liver fat content were found to be variable and not inter-relatable.

In study III, a reference value for a surrogate marker of insulin resistance, HOMA-IR, was determined, its use in the diagnosis of NAFLD evaluated and inter-laboratory variation determined. The study cohorts included two population-based studies, the FINRISK 2007 (n=5024) and the FIN-D2D (n=2849), and a cohort of 368 non-diabetic subjects who underwent measurement of liver fat content using ^{1}H-MRS. In the healthy subjects of FINRISK (n=1167) and FIN-D2D (n=459), the upper reference limits for HOMA-IR (the 95th percentile [95% CI]) were 1.9 (1.8–2.0) and 2.0 (1.9–2.1), respectively. The former corresponded to the optimal HOMA-IR cut-off for diagnosing NAFLD (AUROC 0.88, sensitivity 85%, specificity 80%). The latter matched with a HOMA-IR corresponding to normal liver fat content (5.56%). Inter-laboratory variation of HOMA-IR was determined by simultaneously analysing samples from 10 subjects in 7 European laboratories. The coefficient of variation of HOMA-IR was high, 25%.

In study IV, we determined whether serum pIGFBP-1, which is produced mainly by the liver and regulated by insulin, helps in the estimation of liver fat content independent of other known predictors of liver fat content. Fasting serum pIGFBP-1 was measured in 378 subjects who underwent measurement of liver fat content using ^{1}H-MRS. Serum pIGFBP-1 significantly associated with liver fat content independent of age, waist-to-hip ratio, and fasting ALT, glucose and insulin. This model, '% Liver fat equation', was significantly worse when pIGFBP-1 was removed (p<0.05) and significantly better than liver enzymes ALT and AST (p<0.0001).

In summary, SC adipocyte size is an independent factor contributing to variation in liver fat content. 'Genetic NAFLD' seems not to be characterised by insulin resistance despite larger amounts of liver fat. Definitions of normal liver fat depend on the diagnostic imaging method used and are not inter-related. The upper reference limit of HOMA-IR corresponds to normal liver fat content, but high inter-laboratory variation must be considered. Measurement of pIGFBP-1 may help in non-invasive diagnosis of NAFLD.

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals (I–IV). Articles have been reprinted with the permission of their copyright holders. In addition, some unpublished data are presented.

i. Petäjä EM, Sevastianova K, Hakkarainen A, Orho-Melander M, Lundbom N, Yki-Järvinen H. Adipocyte size is associated with NAFLD independent of obesity, fat distribution and PNPLA3 genotype. *Obesity* 2013;2:1174-9

ii. Petäjä EM, Yki-Järvinen H. Definitions of normal liver fat and the association of insulin sensitivity with acquired and genetic NAFLD – a systematic review. *International Journal of Molecular Sciences* 2016;17:633

iii. Isokuortti E, Zhou Y, Peltonen M, Bugianesi E, Clement K, Bonnefort-Rousselot D, Lacorte J-M, Gastaldelli A, Schuppan D, Schattenberg JM, Hakkarainen A, Lundbom N, Jousilahti P, Männistö S, Keinänen-Kiukaanniemi S, Saltevo J, Anstee QM, Yki-Järvinen H. Use of HOMA-IR in diagnosis of NAFLD – A population-based and inter-laboratory study. *Diabetologia* 2017;60:1873-82

iv. Petäjä EM, Zhou Y, Havana M, Hakkarainen A, Lundbom N, Ihalainen J, Yki-Järvinen H. Phosphorylated IGFBP-1 as a non-invasive predictor of liver fat in NAFLD. *Scientific Reports* 2016;6:24

ABBREVIATIONS

^{1}H-MRS	proton magnetic resonance spectroscopy
AIC	Akaike Information Criteria
ALT	alanine aminotransferase
ARFI	acoustic radiation force impulse
AST	aspartate aminotransferase
AUROC	area under receiver operating characteristic
BCG	bromocrescol green
BCP	bromocrescol purple
BMI	body mass index
CI	confidence interval
CM	chylomicron
CMIA	chemiluminescent microparticle immunoassay
CLIA	chemiluminescence immunoassay
CT	computed tomography
CV	coefficient of variation
DHS	Dallas Heart Study
DNL	*de novo* lipogenesis
EASD	European Association for the Study of Diabetes
EASL	European Association for the Study of the Liver
EASO	European Association for the Study of Obesity
ECLIA	electrochemiluminescence immunoassay
FA	fatty acid
FFA	free fatty acid
FIN-D2D	The Programme for Prevention of Type 2 Diabetes in Finland
FINRISK/DILGOM	The FINRISK 2007 and its sub-study the Dietary Lifestyle and Genetic Determinants of the Development of Obesity and Metabolic syndrome
FLI	Fatty Liver Index
fP	fasting plasma
fS	fasting serum
GGT	gamma-glutamyltransferase
HbA_{1c}	glycated haemoglobin A_{1c}
HCC	hepatocellular carcinoma
HDL	high-density lipoprotein
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
HSI	Hepatic Steatosis Index
HSL	hormone-sensitive lipase
IA	intra-abdominal
IEMA	immunoenzymoassay
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IGF-1	insulin-like growth factor-1
IGFBP-1	insulin-like growth factor binding protein-1
IL	interleukin
ITA	immunoturbidimetric assay
LDL	low-density lipoprotein
LPL	lipoprotein lipase
MBOAT7	membrane bound-O-acyltranserase domain-containing 7
MCP-1	monocyte chemotactic protein-1
MetS	metabolic syndrome

MRE	magnetic resonance elastography
MRI	magnetic resonance imaging
NAFL	non-alcoholic fatty liver
NAFLD	non-alcoholic fatty liver disease
NAS	NAFLD Activity Score
NASH	non-alcoholic steatohepatitis
NPV	negative predictive value
OGTT	oral glucose tolerance test
PDFF	proton density fat fraction
PI	phosphatidylinositol
pIGFBP-1	phosphorylated insulin-like growth factor binding protein-1
PNPLA3	patatin-like phospholipase domain-containing 3 protein
PPAR	peroxisome proliferator-activated receptor
PPV	positive predictive value
RIA	radioimmunoassay
ROC	receiver operating characteristic
SC	subcutaneous
SD	standard deviation
SVF	stromal vascular fraction
TE	transient elastography
TG	triglycerides
TM6SF2	transmembrane 6 superfamily member 2 protein
TNF-α	tumour necrosis factor-α
TZD	thiazolidinedione
US	ultrasound
VLDL	very low-density lipoprotein

1 INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a disease spectrum ranging from simple steatosis (non-alcoholic fatty liver, NAFL) and non-alcoholic steatohepatitis (NASH) to cirrhosis. NAFLD is strongly associated with obesity, type 2 diabetes and the metabolic syndrome (MetS) ('Metabolic NAFLD'), and with increasing prevalence, has become the most common cause of chronic liver disease in the Western countries (Younossi *et al.*, 2011).

'Metabolic NAFLD' can be considered a systemic disease, as it is associated with insulin resistance not only in the liver, but also in adipose tissue and skeletal muscle. A fatty liver is resistant to the action of insulin to suppress production of glucose and triglycerides, leading to hyperinsulinemia, hyperglycaemia, hypertriglyceridemia and low concentrations of high-density lipoprotein (HDL) cholesterol concentrations (Yki-Järvinen, 2014). In adipose tissue, insulin fails to suppress lipolysis, resulting in an increase of free fatty acid (FFA) flux to the liver. Insulin-resistant adipose tissue is characterised by inflammation and altered secretion of adipokines (Hoffstedt *et al.*, 2004). Insulin resistance has also been associated with enlarged fat cells but how adipocyte cell size relates to NAFLD is unknown.

Given that insulin resistance characterises 'Metabolic NAFLD', measurement of insulin sensitivity might be helpful in diagnosing NAFLD in clinical practice. The homeostasis model assessment of insulin resistance (HOMA-IR) is calculated by multiplying the fasting glucose concentration with that of insulin (Matthews *et al.*, 1985). This measure is an excellent surrogate of insulin resistance in non-diabetic subjects in whom insulin secretion readily responds to increases in fasting glucose concentrations. The reference values for HOMA-IR and particularly its relationship to liver fat content have not been established nor have insulin assays been standardised across different laboratories.

In addition to glucose, the liver produces most serum proteins. Ideally one would like to identify proteins which are regulated by insulin and which are exclusively produced by the liver. Our laboratory has previously shown that one such factor is the insulin-like growth factor binding protein-1 (IGFBP-1) (Kotronen, Lewitt, *et al.*, 2008). It is unknown whether measuring its major circulating form, phosphorylated IGFBP-1 (pIGFBP-1), improves diagnosis of 'Metabolic NAFLD' compared to routinely available markers.

In 2008, a common variant in the patatin-like phospholipase domain-containing 3 (*PNPLA3*) (rs738409 [G], encoding I148M) was found to predispose to NAFLD (Romeo *et al.*, 2008). Individuals carrying this genetic variant have increased liver fat content, but seem not to be insulin resistant and therefore not at increased risk of type 2 diabetes or cardiovascular diseases. One would therefore predict that this variant might influence the relationship between liver fat content and HOMA-IR, but this has not been studied. This may also be the case for another recently described genetic risk factor for NAFLD i.e. rs58542926 [T], which encodes E167K in the transmembrane 6 superfamily member 2 (*TM6SF2*) gene (Kozlitina *et al.*, 2014).

The studies in the present series were undertaken to better understand the pathogenesis of NAFLD and to improve currently available diagnostic tools. In study I, we were particularly interested in understanding whether subcutaneous adipocyte size is associated with liver fat independent of other factors, such as age, gender and obesity. Studies II–IV addressed diagnostic tools. We systematically reviewed the literature

regarding existing definitions of normal liver fat, and associations between insulin sensitivity and 'Genetic NAFLD' (II). We wished to define reference values for HOMA-IR based on two population-based cohorts and the relationship of HOMA-IR to liver fat content in subjects whose liver fat content had been measured using proton magnetic resonance spectroscopy (^{1}H-MRS), the state-of the art method to quantify liver fat content (III). Finally, we explored the utility of measuring pIGFBP-1 in the diagnosis of NAFLD (IV).

2 REVIEW OF THE LITERATURE

2.1. INTRODUCTION TO NAFLD

2.1.1. Definitions

NAFLD is defined as hepatic steatosis not caused by excess alcohol intake (>30g/day in men and >20g/day in women), hepatitis B or C, autoimmune hepatitis, iron overload, or use of drugs or toxins (Chalasani *et al.*, 2012). NAFLD is in fact a disease spectrum that ranges from simple steatosis and NASH to cirrhosis (Chalasani *et al.*, 2012). NASH is characterised, in addition to steatosis, by ballooning necrosis, mild inflammation and possibly fibrosis, and can only be diagnosed by liver biopsy (Brunt *et al.*, 2011).

Although NAFLD frequently coexists with obesity, insulin resistance, type 2 diabetes and the and other conditions associated with the MetS ('Metabolic NAFLD') (Anstee and Day, 2013), common genetic forms of NAFLD also exist ('Genetic NAFLD'). A variant in patatin-like phospholipase domain-containing 3 (*PNPLA3*) (rs738409 [G], encoding I148M) increases the risk of NAFL, NASH and fibrosis ('PNPLA3 NAFLD') (Romeo *et al.*, 2008; Sookoian and Pirola, 2011). Genetic variation in transmembrane 6 superfamily member 2 (*TM6SF2*) (rs58542926 [T], encoding E167K) is also associated with higher liver fat content and increased risk of NASH and cirrhosis ('TM6SF2 NAFLD') (Dongiovanni *et al.*, 2015; Kozlitina *et al.*, 2014). Most recently, genetic variation in membrane bound-O-acyltranserase domain-containing 7 (*MBOAT7*) (rs641738 [T]) has been associated with increased risk of steatosis, NASH and fibrosis ('MBOAT7 NAFLD') (Mancina *et al.*, 2016).

2.1.2. Prevalence and significance

NAFLD has become the most common cause for chronic liver disease, causing 47% to 75% of chronic liver disease in 1988 to 2008, respectively (Younossi *et al.*, 2011). According to a recent meta-analysis, the global prevalence of NAFLD is 25% (Younossi *et al.*, 2016). The prevalence, however, is highly variable around the world, as it ranges from 13% in Africa, 24% in Europe and North America, 28% in Asia, 30% in South America to 32% in the Middle East (Younossi *et al.*, 2016). NAFLD is found in 60% to 70% of patients with type 2 diabetes (Targher *et al.*, 2007; Williamson *et al.*, 2011) and up to 85% of morbidly obese subjects undergoing bariatric surgery (Beymer *et al.*, 2003; Gholam *et al.*, 2002; Morita *et al.*, 2015). NAFLD has become the third most common cause of liver transplantation in the United States (Marchesini and Mazzotti, 2015).

The prevalence of NASH ranges from 3% to 6% (Anstee and Day, 2013; Vernon *et al.*, 2011). NASH is found in 10% to 20% of subjects with NAFLD (Adams *et al.*, 2005; Vernon *et al.*, 2011). In longitudinal studies, fibrosis stage, but no other features of NASH, is independently associated with overall mortality, liver transplantation and other liver-related events (Angulo *et al.*, 2015; Ekstedt *et al.*, 2015). In a 33-year follow-up, subjects with NAFLD had increased mortality compared to a reference population, and a higher risk of death from cardiovascular disease, infectious diseases, cirrhosis and hepatocellular carcinoma (HCC) (Ekstedt *et al.*, 2015). Interestingly, while the activity of NASH did not predict mortality, fibrosis stage did predict both overall and disease-specific mortality (Ekstedt *et al.*, 2015).

Unlike previously thought, simple steatosis can also progress to NASH and clinically significant fibrosis (McPherson *et al.*, 2015; Pais *et al.*, 2011; Wong *et al.*, 2010).

During a 5-year follow-up, NASH developed in 2% to 3% of those with NAFL, and cirrhosis developed in 8% of those with NASH (Adams *et al.*, 2005; Vernon *et al.*, 2011). Fibrosis progression is slower in NAFL compared to NASH, as fibrosis progressed one stage in 14.3 years in subjects with NAFL, compared to one stage in 7.1 years in subjects with NASH (Singh *et al.*, 2015).

NAFLD increases the risk of type 2 diabetes, chronic kidney disease and cardiovascular disease (Anstee and Day, 2013). NAFLD also increases the risk of HCC, especially in patients with cirrhosis (Michelotti *et al.*, 2013) but also without cirrhosis (Ertle *et al.*, 2011). According to the National Cancer Registry in Finland, there are 470 to 500 new liver cancer diagnoses yearly (Syöpärekisteri, 2016). HCC accounts for 85% to 90% of all primary liver cancers (El-Serag and Rudolph, 2007), and assuming NAFLD is the cause of HCC in 5% to 20% of cases in Western countries (Michelotti *et al.*, 2013), there are approximately 16 to 90 new NAFLD-related HCC diagnoses yearly in Finland.

2.1.3. Risk factors for NAFLD

2.1.3.1. Gender

Overall, men are more susceptible to NAFLD than women (Browning *et al.*, 2004; Pan and Fallon, 2014; Williams *et al.*, 2011; Zhu *et al.*, 2015). If classified according to body mass index (BMI), lean subjects (BMI <25 kg/m^2) with NAFLD are more often women, whereas overweight (BMI 25–30 kg/m^2) and obese (BMI <30 kg/m^2) subjects are more often men (Younossi *et al.*, 2012). For a given BMI, men have higher lean mass as well as more intra-abdominal (IA) and hepatic fat, whereas women have greater subcutaneous (SC) adipose depots (Geer and Shen, 2009). In cases of a similar amount of liver fat, women have more SC adipose tissue and a higher BMI than men (Kotronen, Westerbacka, *et al.*, 2007). The correlation between liver fat and IA fat is similar in men and women, even though women have more SC adipose tissue (Kotronen, Westerbacka, *et al.*, 2007; Westerbacka *et al.*, 2004).

2.1.3.2. Age

The prevalence of NAFLD increases with age (Adams *et al.*, 2005; Ong *et al.*, 2008; Park *et al.*, 2006). In a recent meta-analysis, the prevalence of NAFLD was 22% to 27% in under the age of 50 years and 34% in subjects of 70-79 years of age (Younossi *et al.*, 2016). Older individuals have more risk factors for NAFLD and present with more severe biochemical and histological changes (Frith *et al.*, 2009). Subjects with NAFLD cirrhosis are significantly older than those with milder disease (Frith *et al.*, 2009). Alarmingly, young age does not protect one from NAFLD. Population-based studies have found that the global prevalence of paediatric NAFLD is 3%, and that the prevalence of NAFLD in obese children ranges from 10% to 77% (Barshop *et al.*, 2008).

2.1.3.3. Obesity and the metabolic syndrome

Obesity is strongly associated with NAFLD. In an analysis of liver histology from liver donors, automobile crash victims, autopsy findings and clinical liver biopsies, the prevalence rates of steatosis and NASH have been approximated to be 15% and 3%, respectively, in non-obese subjects; 65% and 20%, respectively, in obese subjects; 85% and 40%, respectively, in morbidly obese subjects (BMI ≥40 kg/m^2) (Fabbrini *et al.*, 2010). A meta-analysis of 12 studies, consisting of 1620 morbidly obese subjects undergoing bariatric surgery, found the prevalence of steatosis, NASH and unexpected cirrhosis to be 91% (range 85–98%), 37% (24-98%) and 1.7% (1–77%), respectively (Machado *et al.*, 2006). On the other hand, a recent meta-analysis

estimated the prevalence of obesity to be 51% and 82% in subjects with NAFLD and NASH, respectively (Younossi *et al.*, 2016).

MetS is a cluster of metabolic abnormalities that are either causes or consequences of insulin resistance, and that coexist commonly in obese subjects (Yki-Järvinen, 2014). The diagnosis of MetS requires at least three of the following criteria: increased fasting plasma (fP)-glucose or diagnosis of type 2 diabetes, hypertriglyceridemia, low concentrations of high-density lipoprotein (HDL) cholesterol, increased waist circumference (ethnicity dependent) or hypertension (Alberti *et al.*, 2009). NAFLD is closely related to all components of the MetS (Adams *et al.*, 2009; Adiels *et al.*, 2008; Kotronen, Westerbacka, *et al.*, 2007; Marchesini *et al.*, 2001; 2003). NAFLD has been shown to predict development of type 2 diabetes in 20 prospective studies independent of age and obesity (Lallukka and Yki-Järvinen, 2016).

NAFLD can also be found in lean individuals, and they are at increased risk of type 2 diabetes and MetS compared to lean subjects without NAFLD (Feng *et al.*, 2014). In a population-based study, compared to lean subjects without NAFLD, lean subjects with NAFLD were more commonly Hispanic, and had type 2 diabetes and hypertension (Younossi *et al.*, 2012). However, compared to obese subjects with NAFLD, lean subjects with NAFLD are younger and present with fewer components of MetS (Younossi *et al.*, 2012). As discussed below, neither 'PNPLA3 NAFLD' (Sookoian and Pirola, 2011) nor 'TM6SF2 NAFLD' (Sookoian *et al.*, 2015) seem to be associated with obesity. These gene variants are therefore of interest when searching for causes of NAFLD in lean subjects with NAFLD.

2.1.3.4. Race and ethnicity

NAFLD is the most frequent in Hispanics and East Asian Indians and least common in African Americans (Browning *et al.*, 2004; Petersen *et al.*, 2006; Schneider *et al.*, 2013; Williams *et al.*, 2011). These differences are in part explained by the difference in frequencies of known risk genotypes such as the PNPLA3 I148M variant (Romeo *et al.*, 2008), as well as differences in body fat distribution and body composition among the different ethnic groups. For example, in cases of similar BMI, Asians have more visceral fat and less lean body mass compared to Caucasians (Dudeja *et al.*, 2001; WHO Expert Consultation, 2004) and Hispanics and Caucasians have more visceral fat than African Americans despite similar BMI and waist circumference (Carroll *et al.*, 2008). Chinese have more liver fat for a given BMI than Caucasians (Xia, Yki-Järvinen, *et al.*, 2016).

2.1.3.5. Dietary factors and physical inactivity

Excess energy intake increases the risk of NAFLD (Bo *et al.*, 2014). It seems that particularly diets containing excess carbohydrates or saturated fatty acids (FAs) increase liver fat content and deteriorate insulin sensitivity (Yki-Järvinen, 2015). Saturated FAs seem to be more harmful to the liver than polyunsaturated FAs (Bjermo *et al.*, 2012; Parks *et al.*, 2017).

Regarding physical activity, a population-based study including 3056 subjects examined the relationship between NAFLD and physical activity assessed by accelerometer readings over 7 days. Subjects with NAFLD (diagnosed using the Fatty Liver Index (Bedogni *et al.*, 2006)) were less physically active than those without NAFLD (Gerber *et al.*, 2012). On the other hand, lifestyle modifications — weight reduction and/or increased physical activity — consistently reduced liver fat and improved glucose control and insulin sensitivity in 23 lifestyle intervention studies (Thoma *et al.*, 2012). The data on improvement of histopathology is still

scarce, but suggestive that steatohepatitis might improve (Thoma *et al.*, 2012; Vilar-Gomez *et al.*, 2015).

2.1.3.6. Genetic risk factors of NAFLD

The association between NAFLD and genetic variation in *PNPLA3* at rs738409 [G], encoding I148M, was first reported in 2008 (Romeo *et al.*, 2008) and has since been replicated multiple times (Kotronen, Johansson, *et al.*, 2009; Sookoian and Pirola, 2011; Xu *et al.*, 2015; Zhang *et al.*, 2015). The gene variant is common with an allele frequency ranging from 20% to 50% depending on ethnicity (Romeo *et al.*, 2008). In a meta-analysis, carriers of the PNPLA3 I148M variant had 73% more liver fat, a 3.3-fold higher risk of NASH and a 3.3-fold greater risk of developing fibrosis than the non-carriers of the variant (Sookoian and Pirola, 2011). A meta-analysis of 12 Asian studies found the risk of NAFLD to be increased by 2-fold in carriers compared to non-carriers (Zhang *et al.*, 2015). Recent meta-analyses have also shown that the PNPLA3 I148M variant increases the risk of liver cirrhosis by 2-fold (Shen *et al.*, 2015) and the risk of HCC by 2-fold (Trépo *et al.*, 2014).

In 2014, genetic variation in *TM6SF2* at rs58542926, encoding E167K, was found to increase the risk of NAFLD independent of genetic variation in *PNPLA3* at rs738409, degree of obesity and alcohol intake (Kozlitina *et al.*, 2014). The allele frequency of the TM6SF2 E167K variant is less than that of the PNPLA3 I148M variant, ranging from 7.2% in Caucasians and 4.7% in Hispanics to 3.4% in African Americans (Kozlitina *et al.*, 2014). A meta-analysis found carriers of TM6SF2 E167K to have a 2-fold higher risk of developing NAFLD than non-carriers (Pirola and Sookoian, 2015). In addition to increasing the risk of steatosis, several studies have found the TM6SF2 E167K variant to also confer an increased risk of NASH, liver fibrosis and cirrhosis compared to non-carriers (Dongiovanni *et al.*, 2015; Eslam *et al.*, 2016; Liu *et al.*, 2014).

Most recently, in 2016, a single nucleotide polymorphism C>T in *MBOAT7* at rs641738, was shown to increase the risk of steatosis in a population-based cohort of 2736 subjects, and risk of steatosis, NASH and liver fibrosis in 1149 subjects who had undergone liver biopsy (Mancina *et al.*, 2016). In the population-based multiethnic cohort, the risk of NAFLD was increased in Caucasians but not in African or Hispanic Americans (Mancina *et al.*, 2016). The variant allele is common, with an allele frequency ranging from 33% to 46%, depending on ethnicity (Mancina *et al.*, 2016).

2.2. PATHOGENESIS OF NAFLD

2.2.1. 'Metabolic NAFLD'

As discussed above, the prevalence of NAFLD is closely linked to features of MetS, obesity and insulin resistance (Lazo *et al.*, 2013). Insulin resistance can be defined as a condition where the response to one or several actions of insulin is blunted. As discussed below, insulin resistance is an essential feature of 'Metabolic NAFLD' (Yki-Järvinen, 2014). NAFLD is associated with insulin resistance at the level of the whole body (Marchesini *et al.*, 2001), in the liver (Seppälä-Lindroos *et al.*, 2002), in skeletal muscle (Bugianesi, Gastaldelli, *et al.*, 2005; Marchesini *et al.*, 2001) and in adipose tissue (Bugianesi, Gastaldelli, *et al.*, 2005; Kotronen, Vehkavaara, *et al.*, 2008; Ryysy *et al.*, 2000).

2.2.1.1. Normal glucose metabolism

Maintenance of normal glucose homeostasis relies on simultaneous regulation of glucose production and utilisation in the fasted and postprandial state. After an overnight fast, the liver is the exclusive source of glucose, which is

either newly synthetized via gluconeogenesis or released from hepatic glycogen via glycogenolysis (Owen *et al.*, 1969). The main action of insulin after an overnight fast is to restrain hepatic glucose production (Yki-Järvinen, 1993). Glucagon counteracts this inhibitory effect of insulin (Cherrington *et al.*, 1987). After an overnight fast, glucose is utilised mainly by the brain in a non-insulin dependent way (DeFronzo *et al.*, 1983).

Under postprandial conditions, the combined effects of hyperglycaemia, increase in insulin and decrease in glucagon secretion suppress endogenous glucose production and stimulate glucose uptake by the splanchnic organs (gut, liver) and utilisation by the peripheral tissues (Kelley *et al.*, 1988). The main energy source of insulin-dependent tissues after a meal is glucose rather than FFAs, due to insulin-induced inhibition of lipolysis and stimulation of glucose uptake (Yki-Järvinen, 1993). After an oral glucose load, approximately one third is taken up by splanchnic tissues, and one fourth by muscle tissue and the brain (Kelley *et al.*, 1988). 37% of the oral glucose load is oxidised, mainly by brain and muscle tissues, and 63% is stored, mainly by splanchnic and muscle tissues (Kelley *et al.*, 1988).

2.2.1.2. Insulin resistance of glucose metabolism in the liver

Liver fat content is inversely correlated with suppression of hepatic glucose production, a marker of hepatic insulin resistance, in subjects with (Kotronen, Vehkavaara, *et al.*, 2008) and without (Kotronen, Vehkavaara, *et al.*, 2007; Marchesini *et al.*, 2001; Ryysy *et al.*, 2000; Seppälä-Lindroos *et al.*, 2002) type 2 diabetes. This correlation remains significant after adjustment for BMI and waist-to-hip ratio (Seppälä-Lindroos *et al.*, 2002).

2.2.1.3. Insulin resistance of glucose metabolism in skeletal muscle

The ability of insulin to stimulate glucose uptake in skeletal muscle is impaired in subjects with NAFLD compared to subjects without NAFLD (Bugianesi, Gastaldelli, *et al.*, 2005; Kotronen, Seppälä-Lindroos, *et al.*, 2008; Marchesini *et al.*, 2001), and the correlation is inverse and linear with increasing liver fat content (Korenblat *et al.*, 2008).

2.2.1.4. Normal lipid metabolism

After an overnight fast, triglycerides in adipose tissue are hydrolysed by hormone-sensitive lipase (HSL) and other lipases. FFAs are released into the plasma and taken up by the liver and other tissues such as skeletal and heart muscles. In the liver, FAs are preferentially oxidised (Frayn *et al.*, 2006). FFAs are the major substrate for intrahepatocellular triglycerides after an overnight fast (Lambert and Parks, 2012). Insulin inhibits lipolysis by inhibiting HSL and the oxidation FAs (Frayn *et al.*, 2006).

After a meal, dietary fat is hydrolysed in the gut lumen by intestinal lipases, taken up by enterocytes and repackaged into chylomicron (CM) lipoprotein particles. These are secreted into the lymph and then enter the circulation (Lambert and Parks, 2012). Most triglycerides in CM (CM-TG) are hydrolysed by lipoprotein lipase (LPL) in peripheral tissues, and stored as triglycerides in adipose tissue after re-esterification. The remaining CM-remnants are taken up by the liver (Frayn *et al.*, 2006). A portion of FAs released in hydrolysis of CM-TG spill over into plasma and provide substrates for liver triglyceride synthesis (Lambert and Parks, 2012).

Hepatic FAs are derived from dietary FAs from CM-remnants, lipolysis of peripheral triglycerides from adipose tissue or *de novo* lipogenesis (DNL) in the liver (Frayn *et al.*, 2006). In the liver, FAs are diverted

towards β-oxidation, ketone body production or triglyceride and other glycerolipid synthesis. A part of stored triglycerides is secreted into circulation in very low-density lipoprotein (VLDL) particles. In the liver, uptake of FFAs seems to depend mainly on delivery (Frayn *et al.*, 2006). Secretion of VLDL particles is acutely suppressed by insulin (Adiels *et al.*, 2007).

2.2.1.5. Insulin resistance of lipid metabolism in the liver

Excess triglyceride storage in the liver may be a consequence of excess FFAs entering the liver, increased DNL or impaired FFA oxidation and ketogenesis. Defects in VLDL synthesis and secretion could also contribute to steatosis as in familial hypobetalipoproteinemia (Amaro *et al.*, 2010). These abnormalities in triglyceride handling are not necessary or sufficient to cause insulin resistance, as triglycerides themselves are inert, but rather excessive accumulation of FA-derived lipid metabolites is lipotoxic (Matsuzaka and Shimano, 2011; Samuel and Shulman, 2012).

As discussed below, increased FFA release from adipose tissue contributes to increased liver fat content. In 'Metabolic NAFLD', studies using stable isotopes have shown that FAs are also produced in excess via DNL (Diraison *et al.*, 2003; Donnelly *et al.*, 2005; Lambert *et al.*, 2014). In fasted, lean individuals, less than 5% of FAs of triglycerides in VLDL (VLDL-TG) originate from DNL as compared to subjects with NAFLD, in whom up to 20% of the FAs in VLDL-TG originate from DNL (Diraison *et al.*, 2003; Donnelly *et al.*, 2005). DNL produces saturated FAs (Aarsland and Wolfe, 1998), which have been shown to be increased in the circulation of subjects with NAFLD (Westerbacka *et al.*, 2010: Orešič *et al.*, 2013). In insulin-resistant subjects with NAFLD, the liver lipidome is markedly enriched with saturated and monounsaturated FAs, as well as dihydroceramides and ceramides, which are synthesised from saturated FAs (Luukkonen, Zhou, Sädevirta, *et al.*, 2016). Ceramides are bioactive and impair insulin signalling (Summers, 2006).

Secretion of VLDL-TG serves as a mechanism for liver to reduce liver fat content. Hepatic insulin resistance is characterised by a defect in insulin inhibition of VLDL production. In subjects with NAFLD, the liver overproduces triglyceride-rich VLDL particles in the fasting state (Adiels *et al.*, 2006) and during hyperinsulinemia (Adiels *et al.*, 2007). This leads to hypertriglyceridemia and low HDL cholesterol concentration (Syvänne and Taskinen, 1997).

Indirect measures of hepatic lipid oxidation, assessed by plasma 3-hydroxybutyrate concentration, suggest that hepatic lipid oxidation is unchanged in subjects with NAFLD compared to subjects without NAFLD in the fasted state and in euglycaemic hyperinsulinemia (Kotronen, Seppälä-Lindroos, *et al.*, 2009).

2.2.1.6. Insulin resistance in adipose tissue

Adipose tissue triglyceride storage is stimulated and mobilisation suppressed by insulin. Suppression of adipose tissue lipolysis is extremely sensitive to insulin (Nurjhan *et al.*, 1986). In insulin-resistant adipose tissue, release of FFAs and glycerol into circulation is increased. Insulin-mediated suppression of lipolysis is impaired in overweight subjects with NAFLD compared to lean, age- and gender-matched, healthy controls (Marchesini *et al.*, 2001). The ability of insulin to suppress lipolysis closely correlates with liver fat content in subjects with and without type 2 diabetes (Gastaldelli *et al.*, 2007; Kotronen, Seppälä-Lindroos, *et al.*, 2008; Kotronen, Vehkavaara, *et al.*, 2008). Thus, increase in adipose tissue lipolysis causes an increase in the supply of FFA to the liver.

Gene expression of FAT/CD36, which increases FFA uptake from plasma to cells, is increased in the liver and skeletal muscle, but decreased in adipose tissue of obese subjects with NAFLD compared to obese subjects without NAFLD (Fabbrini *et al.*, 2009; Greco *et al.*, 2008). This could contribute to FFA storage outside adipose tissue.

Abdominal obesity, e.g. accumulation of fat in the visceral or IA fat depot, is better correlated with hepatic steatosis and MetS than with overall obesity (Kotronen, Westerbacka, *et al.*, 2007). Liver fat content is significantly and positively correlated with IA and SC adipose tissue volume (Kotronen, Westerbacka, *et al.*, 2007). According to the 'portal hypothesis', visceral adipose tissue releases excess FFA into the portal vein and exposes the liver to high FFA concentrations (Frayn *et al.*, 2006). However, hepatic venous catheterisation studies have shown that only 5% and 20% of splanchnic FFA uptake originate from visceral fat in lean and obese subjects, respectively, and the contribution increases as visceral fat mass increases (Nielsen *et al.*, 2004). Thus, the main source of FFA entering the liver is the peripheral SC fat depot (Nielsen *et al.*, 2004).

2.2.1.6.1. Adipose tissue inflammation

Adipose tissue consists mostly of adipocytes, but also includes a stromal-vascular fraction (SVF) of preadipocytes, fibroblasts, vascular endothelial cells and a variety of immune cells. While the main role of adipose tissue is to store energy as lipids, it is also an active endocrine organ that secretes various hormones, such as leptin and adiponectin, and a variety of bioactive peptides, that are known as adipokines (Kershaw and Flier, 2004). They act both locally in an autocrine/paracrine fashion and systemically in an endocrine fashion (Kershaw and Flier, 2004). In addition to these efferent signals, adipose tissue expresses a multitude of receptors that allows it to respond to hormonal and neural stimuli (Kershaw and Flier, 2004). At least 600 adipokines have been identified (Dahlman *et al.*, 2012; Lehr *et al.*, 2012). They regulate immune responses, inflammation, glucose and lipid metabolism, insulin sensitivity, appetite and satiety, and other biological processes (Blüher 2012). Two types of adipose tissue exist – the white adipose tissue which stores energy, and brown adipose tissue which generates body heat (Virtanen and Nuutila P, 2011). This thesis focuses on white adipose tissue.

Adipose tissue inflammation has been proposed to play an important role in obesity-related insulin resistance (Blüher, 2016; Heilbronn and Campbell, 2008). Many of the adipokines, including monocyte chemotactic protein-1 (MCP-1), tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6 and IL-8, have been reported to promote insulin resistance (Lackey and Olefsky, 2016). In 2003, Weisberg et al. found, first in mice and subsequently in humans, that the number of macrophages in adipose tissue is increased in obesity (Weisberg *et al.*, 2003). Along with this, it has been shown in mice that obesity induces a phenotypic switch in macrophages from an anti-inflammatory M2 polarisation state to a pro-inflammatory M1 polarisation state (Lumeng *et al.*, 2007). Accumulation of M1 macrophages in adipose tissue has been shown to result in secretion of a variety of pro-inflammatory cytokines, such as TNF-α and IL-6, as well as chemokines, such as MCP-1, that can exert local paracrine actions to decrease insulin signalling (Lackey and Olefsky, 2016) and potentially induce adipose tissue inflammation and insulin resistance (Shoelson *et al.*, 2007). These pro-inflammatory factors may leak out of adipose tissue into the circulation and promote insulin resistance in other peripheral tissues (Osborn and Olefsky, 2012). In contrast, it has been shown in mice that M2-polarised macrophages

participate in the remodelling of adipose tissue, including the clearance of dead or dying adipocytes and the recruitment and differentiation of adipocyte progenitors (Lee *et al.*, 2013).

In humans, the number of macrophages is increased in the adipose tissue of obese subjects, as compared to lean subjects, as well as in the adipose tissue of subjects with NAFLD, as compared to that of weight-matched subjects without NAFLD (Kolak *et al.*, 2007). Weight loss decreases macrophage infiltration and pro-inflammatory gene expression in the adipose tissue of obese subjects (Clément *et al.*, 2004). Furthermore, in a study of 113 morbidly obese subjects undergoing bariatric surgery, subjects with NAFLD and NASH have an increased expression of genes that regulate inflammation in their visceral and SC adipose tissue, and increased circulating cytokines and chemokines such as TNF-α, IL-8 and MCP-1 (du Plessis *et al.*, 2015).

2.2.1.6.2. Adipocyte cell size

In obesity, adipose tissue expands by increasing the number of adipocytes (hyperplasia) or the size of the adipocytes (hypertrophy), or a combination of both (Arner and Spalding, 2010). Hypertrophy is characteristic of all overweight and obese subjects whereas hyperplasia correlates more strongly with the severity of obesity and is most marked in morbidly obese individuals (Arner and Spalding, 2010; Hirsch and Batchelor, 1976). The relationship between SC adipocyte size and body fat mass is curvilinear in men and women (Spalding *et al.*, 2008). It has been suggested that there is a 'critical adipocyte size' that would trigger a subsequent increase in adipocyte number (Arner and Spalding, 2010). This could be because the capacity of adipocytes to store lipids is limited (Virtue and Vidal-Puig, 2010). When this 'critical adipocyte size' limit is reached, the tissue must generate more adipocytes to be able to expand further (Hirsch and Batchelor, 1976; Virtue and Vidal-Puig, 2010). After weight loss, adipocyte volume decreases, but the number of adipocytes fails to decrease (Spalding *et al.*, 2008).

In earlier *in vitro* studies, large adipocytes were less insulin sensitive than small adipocytes (Salans *et al.*, 1973; Smith, 1971). Adipocyte size is also an important determinant of the expression and secretion of several pro-inflammatory adipokines, as larger adipocytes secrete higher amounts of pro-inflammatory mediators (leptin, IL-6, IL-8, MCP-1) and less of anti-inflammatory or insulin sensitizing factors such as adiponectin and IL-10 (Skurk *et al.*, 2007). Larger adipocytes are also more prone to store lipophilic toxins, which may increase intracellular stress, autophagy and apoptosis (Haim *et al.*, 2015; Kosacka *et al.*, 2015). With increasing adipocyte size and limited expandability of adipose tissue, local hypoxia may also contribute to adipose tissue inflammation (Trayhurn, 2013).

Cross-sectional human studies have found SC adipocyte size to have a strong positive correlation with adipose tissue inflammation and whole-body insulin resistance independent of body composition or BMI (Lundgren *et al.*, 2007; Maffeis *et al.*, 2007; Weisberg *et al.*, 2003). Increased SC adipocyte size has been shown to be associated with metabolic impairments such as dyslipidemia (Rydén *et al.*, 2014), hypertension (Ledoux *et al.*, 2009) and markers of insulin resistance (Arner and Spalding, 2010; Björntorp and Sjöström, 1971; Cotillard *et al.*, 2014; Lundgren *et al.*, 2007; Maffeis *et al.*, 2007) independent of body composition (BMI or body fat percentage). Reports on correlation between SC adipocyte size and insulin resistance in subjects with type 2 diabetes

are contradictory. Lundgren *et al.* found no correlation between SC adipocyte size and insulin resistance in 49 subjects with type 2 diabetes (Lundgren *et al.*, 2007), but did find that the diabetic subjects had larger SC adipocyte size than 83 BMI-matched non-diabetic subjects. SC adipocyte size correlated positively with BMI. Pasarica *et al.* found that 41 diabetic subjects had a significantly higher mean SC adipocyte size and greater proportion of very large adipocytes than 192 BMI-matched non-diabetic subjects (Pasarica *et al.,* 2009). In contrast, no correlation was observed between SC adipocyte size and BMI in the diabetic subjects (Pasarica *et al.*, 2009).

These data raise the possibility that increased SC adipocyte size influences liver fat content independent of age, gender, obesity, fat distribution or *PNPLA3* genotype. The latter is of interest as, in addition to liver fat (Sookoian and Pirola, 2011), genetic variation in *PNPLA3* may also impact SC adipocyte size (Santoro *et al.*, 2010).

2.2.1.6.2.1. Methods for assessing adipocyte size

Adipocyte size from adipose tissue biopsy is most commonly determined using one of three methods: collagenase digestion, osmium tetroxide fixation or histological analysis.

The most commonly used method for determination of adipocyte size is collagenase digestion, developed by Rodbell, to separate mature adipocytes from the SVF (Rodbell, 1964). In short, adipose tissue is digested by collagenase and mature adipocytes are separated from the SVF by floatation in an aqueous solution. Adipocyte size is determined by calculating the mean size of 100 to 300 adipocytes with a phase-contrast microscope. A limitation is that small adipocytes may not float as easily as the larger cells due to their lower lipid content (Ashwell *et al.*, 1976), and adipocytes may break in unfixed tissue (Ashwell *et al.*, 1976); however, the use of adenosine in the solution has minimized the bias.

Osmium tetroxide fixation and analysis using the Multisizer Counter was introduced by Hirsch (Hirsch and Gallian, 1968). Osmium tetroxide fixes intracellular lipids and allows staining of very fragile cell types. In short, adipose tissue is digested by collagenase and thereafter fixed with osmium tetroxide, or simultaneously if collidine-HCl solution is used instead of collagenase for digestion. Adipocytes are analysed with a counter that measures cell size by fluctuation of electrical resistance. A wider distribution of adipocyte size may be analysed (Etherton *et al.*, 1977). Even though very small adipocytes may be identified, a threshold value is often used to distinguish between mature adipocytes and artefacts, and multilobular adipocytes may rupture during fixation. This technique is slow and requires handling of osmium tetroxide, which is a hazardous chemical (Ashwell *et al.*, 1976).

Adipocyte size may also be estimated from histological slides. This is the only method for examining global tissue architecture, and immunostaining may be performed simultaneously. Fixation agents are known to cause significant cell shrinkage, and it must be assumed that cell distribution is uniform and that cells occur as perfect spheres that show their largest diameter (Ashwell *et al.*, 1976).

Collagenase digestion and osmium fixation seem to create similar mean values for adipocyte size while histological evaluation results in approximately 15% smaller mean values than the two other methods (Laforest *et al.*, 2015).

2.2.1.6.3. Mechanisms of adipo-hepatic communication: adiponectin

Adiponectin is a protein produced maily by adipocytes (Nigro *et al.*, 2014) and its expression is primarily determined by adipocyte size and insulin sensitivity (Drolet *et al.*, 2012). In the liver, adiponectin has insulin-sensitising, antifibrogenic, and anti-inflammatory properties by acting on hepatocytes, hepatic stellate cells, and hepatic macrophages (Kupffer cells), respectively (Polyzos *et al.*, 2010). Adiponectin knockout mice show impaired insulin signalling in the liver (Yano *et al.*, 2008) and have hepatic insulin resistance (Nawrocki *et al.*, 2006). Moreover, adiponectin knockout mice develop more extensive liver fibrosis compared to wild-type mice, whereas adenovirus-mediated overexpression of adiponectin ameliorates liver damage in wild-type mice (Kamada *et al.*, 2003). In *ob/ob* mice, insulin resistance and liver fat can be significantly improved by overexpression of adiponectin in adipose tissue (Kim *et al.*, 2007).

In humans, an association between adiponectin deficiency and NAFLD has been shown in several studies (Bugianesi, Pagotto, *et al.*, 2005; Koska *et al.*, 2008; Targher *et al.*, 2006). In the Dallas Heart Study (DHS), in which liver fat (^{1}H-MRS) and serum adiponectin were measured in 2215 subjects, adiponectin deficiency was associated with NAFLD independent of obesity (Turer *et al.*, 2012). In a meta-analysis of 27 studies comprising a total of 1545 subjects with NAFLD and 698 controls (Polyzos *et al.*, 2011), healthy subjects had significantly higher serum adiponectin concentrations than those with NAFL, and the subjects with NASH had significantly lower serum adiponectin than the subjects with NAFL (Polyzos *et al.*, 2011). Treatment with PPARγ agonists thiazolidinediones (TZDs), such as pioglitazone and rosiglitazone, increases serum adiponectin by 2- to 3-fold (Bajaj *et al.*, 2004; Tiikkainen *et al.*, 2004). Increase in serum adiponectin correlates closely with decrease in liver fat and with improvement of hepatic insulin resistance (Bajaj *et al.*, 2004; Tiikkainen *et al.*, 2004). Adiponectin regulates ceramide metabolism by upregulating ceramidase, the enzyme that degrades ceramide to sphingosine, and adiponectin deficiency has also been shown to increase ceramide synthesis (Chavez and Summers, 2012). Indeed, adiponectin concentration correlates inversely with hepatic ceramide concentrations in the human liver (Luukkonen, Zhou, Sädevirta, *et al.*, 2016).

2.2.1.7. Insulin clearance and its impact on fasting insulin concentration

Approximately 80% of endogenously secreted insulin and 50% of intravenously infused insulin is cleared by the liver (Ferrannini *et al.*, 1983). Insulin clearance decreases in proportion to liver fat content, and insulin clearance and liver fat content are both independent determinants of the fasting insulin concentration (Kotronen, Vehkavaara, *et al.*, 2007). In 80 non-diabetic subjects whose liver fat content ranged from 0.4% to 41% as measured using ^{1}H-MRS, liver fat content and insulin clearance accounted for 38% and 42% of the variation in fasting serum (fS)-insulin concentrations, respectively (Kotronen, Vehkavaara, *et al.*, 2007). Both liver fat and insulin clearance were independent determinants of fS-insulin concentrations and together accounted for 53% of its variation. Increased liver fat is also associated with impaired insulin clearance in subjects with type 2 diabetes (Kotronen, Seppälä-Lindroos, *et al.*, 2008).

2.2.2. 'Genetic NAFLD'

2.2.2.1. The PNPLA3 I148M variant

In vitro studies and studies in experimental animals suggest two distinct mechanisms for the function of the PNPLA3 I148M variant. The gene variant

has been shown to disrupt intrahepatocellular hydrolysis of triglycerides, resulting in accumulation of triglycerides in hepatocytes (He *et al.*, 2010; Huang *et al.*, 2011). The variant has also been shown to act as a lysophosphatidic acid-acyltransferase and activate triglyceride synthesis to a greater extent from long unsaturated FA containing coenzyme A than from saturated FA containing coenzyme A (Kumari *et al.*, 2012). *In vivo*, the increase in liver fat in carriers of PNPLA3 I148M is due to polyunsaturated triglycerides, unlike in 'Metabolic NAFLD', in which saturated triglycerides and insulin resistance-inducing ceramides are increased (Luukkonen, Zhou, Sädevirta, *et al.*, 2016).

In 22 out of 24 studies, 'PNPLA3 NAFLD' is not associated with features of the MetS, such as hypertriglyceridemia, hyperglycaemia or low HDL cholesterol (Cox *et al.*, 2011; Del Ben *et al.*, 2014; Hyysalo *et al.*, 2014; Kantartzis *et al.*, 2009; Kitamoto *et al.*, 2013; Kotronen, Johansson, *et al.*, 2009; Li *et al.*, 2012; Lin *et al.*, 2011; Musso *et al.*, 2015; Park *et al.*, 2015; Petit *et al.*, 2010; Romeo *et al.*, 2008; Romeo, Sentinelli, Cambuli, *et al.*, 2010; Romeo, Sentinelli, Dash, *et al.*, 2010; Scorletti *et al.*, 2015; Sookoian *et al.*, 2009; Speliotes *et al.*, 2011; Valenti, Alisi, *et al.*, 2010; Verrijken *et al.*, 2013; Wagenknecht *et al.*, 2011; Wang *et al.*, 2011; Xia, Ling, *et al.*, 2016) or inflamed adipose tissue (Lallukka *et al.*, 2013). In a study of morbidly obese subjects undergoing bariatric surgery, PNPLA3 I148M variant was paradoxically associated with increased risk of type 2 diabetes, but lower concentration of circulating triglycerides (Palmer *et al.*, 2012). In a study including 279 overweight or obese adolescents, the prevalence of MetS was higher in the homozygous carriers of the PNPLA3 I148M variant than in the non-carriers (9.2% vs. 5%, respectively, $p<0.05$ for comparison) (Mangge *et al.*, 2015). Liver fat was not measured in these studies. There are no systematic reviews addressing the association of insulin resistance to 'PNPLA3 NAFLD'.

2.2.2.2. The TM6SF2 E167K variant

The exact molecular mechanism by which the TM6SF2 E167K variant increases the risk of NAFLD is still unclear. *In vitro* studies suggest that lipidation of VLDL particles is decreased (Smagris *et al.*, 2016), leading to impaired VLDL secretion and increased liver fat content (Mahdessian *et al.*, 2014). Reducing *Tm6sf2* transcripts in the mouse liver using recombinant adeno-associated viral vectors expressing short hairpin RNAs (Kozlitina *et al.*, 2014) and knocking out *TM6SF2* in mice (Smagris *et al.*, 2016) causes steatosis. In the latter study, plasma VLDL-TG concentration decreased markedly (Smagris *et al.*, 2016). In another study, inhibition of *TM6SF2* by small interfering RNA also decreased the export of triglyceride-rich lipoproteins and lipid droplet content in human hepatoma cells (Huh7 and HepG2) (Mahdessian *et al.*, 2014).

In vivo studies in humans have shown that carriers of the TM6SF2 E167K variant have a 2.1-fold higher risk of NAFLD, while lower circulating total and low-density lipoprotein (LDL) cholesterol, and triglyceride concentrations than the non-carriers (Pirola and Sookoian, 2015). Carriers of the E167K variants seem to be protected against cardiovascular disease (Dongiovanni *et al.*, 2015; Pirola and Sookoian, 2015). No systematic review on the association of insulin resistance and 'TM6SF6 NAFLD' exists.

2.2.2.3. The MBOAT7 rs641738 C>T variant

Little is known about the function of the recently discovered *MBOAT7* variant. MBOAT7 functions as an acyltransferase that catalyses acyl-chain remodelling of phosphatidylinositols (PIs). *In vitro* in

mice, knockout of *MBOAT7* affects concentrations of hepatic polyunsaturated PIs (Anderson *et al.*, 2013). The MBOAT7 rs641738 T allele was found to be associated with lower protein expression in the liver, and in changes in plasma PI species (decrease in PI(36:4) and PI(38:3), but an increase in PI(40:5)), but there was no association in other lipid classes (Mancina *et al.*, 2016). Similar changes in polyunsaturated PIs were also found in the human liver lipidome in carriers of the MBOAT7 variant allele (Luukkonen, Zhou, Hyötyläinen, *et al.*, 2016).

In the only study to report serum lipid data, there was no difference in total, LDL or HDL cholesterol or in triglyceride concentration or incidence of type 2 diabetes between carriers and non-carries of the T allele (Mancina *et al.*, 2016). No studies have reported data on insulin resistance markers in 'MBOAT7 NAFLD'.

2.3. DIAGNOSIS OF NAFLD

The gold standard for the diagnosis of NAFLD is histological examination of a liver biopsy. As performing a liver biopsy to all the patients with suspected NAFLD is not available in clinical practice and carries the risk of severe complications, non-invasive methods for evaluation of hepatic steatosis have been introduced. Hepatic steatosis can be assessed quantitatively or qualitatively using different imaging methods, such as magnetic resonance imaging (MRI), ^{1}H-MRS, ultrasound (US), and computed tomography (CT).

As NAFLD predicts both complications and mortality on liver-related and metabolic causes, the definition of normal liver fat and the recognition of subjects with NAFLD is important. There are no systematic reviews of the definitions of normal liver fat content using different diagnostic methods and how they correspond to the liver histology.

Different laboratory tests and scores using a combination of biochemical and anthropometric variables have also been introduced to evaluate the presence of NAFLD or the degree of liver fat content.

2.3.1. Histology

A liver biopsy is the gold standard for diagnosis of NASH and for assessment of the degree of fibrosis (Rockey *et al.*, 2009). Liver biopsy is most commonly performed percutaneously using US guidance, but can also be obtained transvenously from the jugular or femoral vein, or during laparoscopy or laparotomy (Rockey *et al.*, 2009). The most common complication of a percutaneous liver biopsy is pain, occurring in up to 84% of patients (Eisenberg *et al.*, 2003). However, following a liver biopsy, more serious complications may ensue, most importantly intraperitoneal bleeding and even death (Piccinino *et al.*, 1986). The mortality rate is less than 1 in every 10 000 biopsies, and is usually related to haemorrhage (Myers *et al.*, 2008; Piccinino *et al.*, 1986).

Steatosis, inflammation and ballooning of hepatocytes are characteristic for NASH. For research purposes, two algorithms are used for evaluating the severity of the disease rather than actually diagnosing NASH. The NAFLD Activitity Score (NAS Score) (Kleiner *et al.*, 2005) takes into account steatosis, inflammation, ballooning and fibrosis. Steatosis is evaluated as the amount of steatotic hepatocytes, using a grading from 0 to 3 (0: <5%; 1: 5%–33% 2: 34%–66%; 3: >67%), and also steatosis location from 0 to 3 (0: zone 3; 1: zone 1; 2: azonal; 3: paracinar). Lobular inflammation is graded from 0 to 3 (0: none; 1: <2; 2: 2–4; 3: >4), chronic portal inflammation from 0 to 2 (0: none; 1: mild; 2: > mild) and ballooning from 0 to 2 (0: none; 1: few; 2: many). Fibrosis is scored from 0 to 3 (stage 0: none; 1a–c: 1a or 1b sinusoidal zone 3 or 1c portal fibrosis; 2: perisinusoidal and

periportal fibrosis without bridging; 3: bridging fibrosis; and 4: cirrhosis). Even though some studies use a NAS score of ≥5 as a surrogate for histologic diagnosis of NASH, it has been shown in a study of 976 biopsies that of those with definite NASH, only 75% had a NAS score of ≥5 (Brunt *et al.*, 2011).

A newer, simpler score, the SAF score (Bedossa *et al.*, 2012; Bedossa *et al.*, 2014) was introduced in 2014. The SAF score is determined from three main histological lesions: steatosis, activity and fibrosis. The steatosis score (S, from S_0 to S_3) indicates the quantities of macrovesicular, not microvesicular, lipid droplets as in the NAS score. The activity grade (A, from A_0 to A_4) is the sum of scores of hepatocyte ballooning (0-2, where 0: normal hepatocytes with cuboidal shape and pink eosinophilic cytoplasm; 1: the presence of clusters of hepatocytes with a rounded shape and pale cytoplasm; 2: the same as 1 but with some enlarged hepatocytes) and lobular inflammation (0–2, where lobular inflammation is defined as the focus of two or more inflammatory cells within a lobule, and the foci are counted at 20 x magnification; 0: none; 1: ≤2 foci per 20 x; 2: >2 foci per 20 x), as A_0 (A=0) is no activity, A_1 (A=1) is mild activity, A_2 (A=2) is moderate activity and A_3 (A≥3) is severe activity. The stage of fibrosis (F) is defined as in the NAS score (F_0–F_4). NAFLD is defined as presence of steatosis in >5% of hepatocytes and NASH by the presence, in addition, of hepatocellular ballooning of any degree and lobular inflammatory infiltrates of any amount.

Liver biopsy represents approximately $1/50000^{th}$ to $1/65000^{th}$ of the liver, and is therefore susceptible to sampling error. A study on 51 paired liver biopsies from patients with NAFLD demonstrated that in 24% of cases, ballooning necrosis would have been missed with only one biopsy, and the fibrosis was understaged in 35% of the cases (Ratziu *et al.*, 2005). In a study of 41 paired biopsies from subjects with NAFLD, the agreement of the steatosis grade between the right and left lobe of liver was excellent (κ=0.88), of fibrosis the agreement moderate (κ=0.51), but only modest for lobular inflammation (κ=0.32) and hepatocyte ballooning (κ=0.20) (Merriman *et al.*, 2006). Inter-observer and intra-observer variability between pathologists is considerable especially for lower fibrosis grades (Bedossa *et al.*, 2014). Grading of steatosis and staging of fibrosis is relatively reliable in terms of inter-observer and intra-observer variability, but worse for lobular inflammation and ballooning (Ratziu *et al.*, 2005; Merriman *et al.*, 2006). Overall inter-observer and intra-observer agreement scores for the diagnosis of NASH are moderate to high at 0.66–0.90 and 0.61–0.62, respectively (Ratziu *et al.*, 2005; Merriman *et al.*, 2006).

2.3.2. Imaging methods

2.3.2.1. Liver fat content

2.3.2.1.1. Proton magnetic resonance spectroscopy (^{1}H-MRS)

^{1}H-MRS is an MRI-based method that enables sampling of a much larger volume fraction of the liver compared to a biopsy, typically a single voxel of 2 x 2 x 2 cm^3 or 3 x 3 x 3 cm^3 (Longo *et al.*, 1993; Szczepaniak *et al.*, 1999). ^{1}H spectra of liver tissue show two dominant signals that reflect liver fat content: peaks of water, and peaks of the methylene and methyl protons of triglycerides (Festi *et al.*, 2013). The resonances from the methylene and methyl protons of triglyceride acyl chains appear between 1.0 and 1.6 ppm (Szczepaniak *et al.*, 1999). The fat-signal fraction represents the ratio of the signal from the protons of triglycerides to the sum of the signals from protons of both free water and triglycerides (Festi *et al.*, 2013). Total hepatic triglyceride content is then calculated by summing the individual lipid peaks in the 0.5 to 3.0 ppm region of the

MR spectrum to obtain the total lipid peak area and dividing this with the sum of the total lipid and the water resonance peaks in 3.0 to 5.5 ppm region (Szczepaniak *et al.*, 2005). ^{1}H-MRS is highly reproducible (Cowin *et al.*, 2008; Machann *et al.*, 2006; Szczepaniak *et al.*, 1999), and has become the gold standard for quantifying steatosis as it is the most accurate method (Bohte *et al.*, 2011). Patients are not exposed to excessive radiation, as they are during CT scanning. However, the method is expensive, requires technical expertise for performance and is not readily available in clinical practice.

2.3.2.1.2. Magnetic resonance imaging (MRI)

Several MR imaging techniques are available. The most widely used is called in-phase (IP) and opposed-phase (OP) imaging, that is also known as chemical shift imaging Dixon technique (Cassidy *et al.*, 2009; Dixon, 1984; Fishbein and Stevens, 2001; Fishbein *et al.*, 1997; Rofsky *et al.*, 1996). This technique is universally available in all modern clinical 1.5 and 3 T systems and is included in most clinical abdominal MR examinations (Kinner *et al.*, 2016). In this method, two sets of images are captured: the IP image in which the water and fat signals are approximately in-phase, and the OP image where the two signals are in opposed-phase. The IP image represents the sum of water and fat signals of the liver and the OP image their difference (Kinner *et al.*, 2016). In a non-steatotic liver, where no fat is present, the liver signal in OP and IP is nearly the same, as only the water contributes to the liver signal. With increasing degree of steatosis, the liver parenchyma becomes darker on the OP images. The liver signal fat fraction is defined as the proportion of the fat signal divided by the total (water + fat) signal.

More recently, advanced MRI techniques (magnitude- and complex data-based) have been developed that eliminate the biases seen with conventional MRI and estimate the proton density fat fraction (PDFF), defined as the fraction of mobile protons of triglycerides relative to those that of the properties of water (Bydder *et al.*, 2008; Hines *et al.*, 2011; Meisamy *et al.*, 2011; Yokoo *et al.*, 2009). MRI-PDFF provides an estimation of liver fat content from all the segments of the liver (Bonekamp *et al.*, 2014). While technical details vary, the common strategy is to acquire imaging data at multiple different echo times and perform time-domain analysis (curve fitting) to estimate the signals originating from triglyceride and water. The MRI-PDFF correlates closely with histological liver fat content (Bonekamp *et al.*, 2014; Hines *et al.*, 2011; Noureddin *et al.* 2013).

2.3.2.1.3. Computed tomography (CT)

CT provides an accurate and reliable visualisation of the entire liver, unlike ^{1}H-MRS and liver biopsy. Hepatic steatosis can be best evaluated in non-enhanced CT images (Siegelman and Rosen, 2001). Tissue fat deposition lowers attenuation, making fatty areas less dense and appear darker than the non-fatty tissues, such as the spleen (Piekarski *et al.*, 1980). Steatosis of the liver can be assessed by the absolute measurement of attenuation values in Hounsfield units (HU), comparing attenuation of the liver parenchyma to that of the spleen or calculating the spleen-to-liver attenuation ratio (Schwenzer *et al.*, 2009). The main advantage of CT imaging is its wide availability and relatively moderate cost, but radiation exposure prevents use of CT for screening purposes (Fierbinteanu-Braticevici, 2010).

2.3.2.1.4. Ultrasound (US)

US is a widely available tool that is less expensive than MRI, ^{1}H-MRS or CT (EASL *et al.*, 2016). Hepatic steatosis appears as a diffuse increase in parenchymal brightness and echogenicity in US images, and is often compared to hypoechogenity of the kidney cortex. A disadvantage of US imaging is its poor sensitivity, especially in obese subjects. In a prospective study comprising 187 obese subjects who were to undergo US imaging before bariatric surgery, the sensitivity of US for diagnosing biopsy-proven steatosis was 49% (Mottin *et al.*, 2004). In a study of 100 consecutive living liver donors, the sensitivity of US to detect 5–10%, 10–19%, 20–30% and >30% steatosis was 12%, 55%, 72%, and 80% (Ryan *et al.*, 2002). Another weakness of ultrasound is its operator-dependency. Three independent, experienced radiologists evaluated liver steatosis in 168 patients, and the examination was repeated after one month. The mean inter- and intra-observer agreement rates for the presence of increased liver fat were 72% and 76% (Strauss *et al.*, 2012). Intra-observer agreement for the severity of fatty liver ranged from 55% to 68% (Strauss *et al.*, 2012).

2.3.2.2. Assessment of fibrosis

Transient elastography (TE) with Fibroscan® (Echosens, Paris, France) (Sandrin *et al.*, 2003) and acoustic radiation force impulse (ARFI) elastography using Siemens AcusonS2000 (Siemens AG, Erlangen, Germany) are US-based techniques for non-invasive assessment of liver stiffness, which is a surrogate marker for liver fibrosis (Bota *et al.*, 2013). A meta-analysis consisting of 13 studies and 1163 subjects who had undergone a liver biopsy concluded that the two methods performed equally well in detection of significant fibrosis and cirrhosis (Bota *et al.*, 2013). For the detection of significant fibrosis (F≥2), the sensitivity was 74% and specificity 83% for ARFI, while for TE the sensitivity was 78% and specificity 84%. For the diagnosis of cirrhosis, the sensitivity was 87% and specificity 87% for ARFI, and, respectively, 89% and 87% for TE.

Magnetic resonance elastography (MRE) is a non-invasive MRI-based technique for quantitative assessment of increased stiffness of the liver parenchyma (Venkatesh *et al.*, 2013; Yin *et al.*, 2007). MRE may be performed simultaneously with MRI-PDFF. A meta-analysis of 11 MRE studies comprising 982 patients found that area under the receiver operating characteristics curve (AUROCs) for MRE staging fibrosis were 0.94, 0.97, 0.96 and 0.97 for F1–F4, respectively, and in 15 ARFI studies comprising 2128 patients 0.82, 0.85, 0.94 and 0.94 for F1–F4, respectively (Guo *et al.*, 2014). The two studies comparing MRE and TE in differentiation of significant fibrosis concluded that MRE is significantly more accurate than TE (Imajo *et al.*, 2016; Park *et al.*, 2017).

2.3.3. Circulating biochemical markers

In 'Metabolic NAFLD', liver fat closely correlates with direct and indirect measures of insulin resistance, liver enzymes and other markers.

2.3.3.1. Measures of insulin resistance

2.3.3.1.1. Fasting insulin

In 271 non-diabetic subjects studied in our laboratory, the correlation coefficient between liver fat measured by ^{1}H-MRS and fS-insulin concentration was 0.61 ($p<0.001$). The relationship between liver fat and fS-insulin remained significant even after adjustment for age, gender and BMI (Kotronen, Westerbacka, *et al.*, 2007). No difference in regression lines or slopes was found between genders. The correlation coefficient was better than that

between liver fat and liver enzymes such as alanine aminotransferase (ALT) (r=0.39, p<0.0001 for women and r=0.33, p<0.0001 in men, significant difference in intercepts) and aspartate aminotransferase (AST) (r=0.27, p=0.0005 for women and r=0.31, p=0.0012 in men, significant difference in intercepts) (Kotronen, Westerbacka, *et al.*, 2007). In subsequent studies, a similar close relationship between liver fat measured with ^{1}H-MRS and fasting insulin has been found. These studies included 42 non-diabetic Americans (r=0.60, p<0.001) (Korenblat *et al.*, 2008), 43 diabetic Americans (r=0.48, p<0.05) (Gastaldelli *et al.*, 2007), 17 Australians (r=0.50, p<0.05) (Chan *et al.*, 2006), 47 non-diabetic Chinese (r=0.41, p<0.05) (Bian *et al.*, 2011), 216 type 2 diabetic Americans (r=0.31; p<0.001) and 136 non-diabetic Americans (r=0.37; p<0.001) (Bril *et al.*, 2017).

When diabetes develops, insulin secretion starts to decrease relative to plasma glucose, which complicates its use as a surrogate for liver fat in the subjects (DeFronzo *et al.*, 1992; DeFronzo, 2009; Gastaldelli, 2011; Lillioja *et al.*, 1988;). Another problem is that insulin analogues are not detected with modern insulin assays (Heurtault *et al.*, 2014). As type 2 diabetic patients' requirements for insulin correlate with their insulin resistance (Ryysy *et al.*, 2000), it would thus be desirable to measure all the insulins.

The intra- and inter-assay coefficient of variation (CV) of a single insulin assay and variation between different insulin assays should be known (Manley *et al.*, 2007). In 1996, the American Diabetes Association task force on standardisation of insulin assays concluded that there is wide inter-assay variation in insulin measurements (Robbins *et al.*, 1996). Manley *et al.* have compared 11 insulin assays and showed that insulin concentrations vary over 2-fold depending on the assay used (Manley *et al.*, 2007). Assay specificity, calibration procedures, specimen types, assay performance, and conversion factors may contribute to inter-assay variation (Manley *et al.*, 2007). Regarding conversion between units from mU/l to pmol/l, the conversion factor varies between 6.0 to 7.46 depending on the assay used, and is yet another source of variation (Manley *et al.*, 2007). Proinsulin cross-reacts with insulin antibodies in some assays, although proinsulin concentrations do not usually exceed 10% of the total insulin measured (Manley *et al.*, 2007). Specific assays are therefore recommended (Wallace and Matthews, 2002).

2.3.3.1.2. HOMA-IR

The homeostasis model assessment of insulin resistance (HOMA-IR) is calculated as the product of fasting glucose (in mmol/l) and insulin (in mU/l) concentrations divided by 22.5 (Matthews *et al.*, 1985). As insulin resistance develops in the liver, glucose concentrations increase because insulin fails to suppress hepatic glucose production in the normal manner (Seppälä-Lindroos *et al.*, 2002). This stimulates beta cells to increase insulin secretion resulting in a combination of hyperglycemia and hyperinsulinemia. This combination increases the product of fasting glucose and insulin, i.e. HOMA-IR.

HOMA-IR provides a good surrogate marker of hepatic insulin resistance as long as glucose tolerance remains non-diabetic. After the development of diabetes, insulin secretion starts to decrease relative to plasma glucose (DeFronzo *et al.*, 1992; DeFronzo, 2009; Gastaldelli, 2011; Lillioja *et al.*, 1988;). Under such conditions, HOMA-IR is no longer a reliable marker of insulin resistance. If there is no endogenous insulin secretion left (type 1 diabetes), insulin measurement does not reflect insulin resistance, and direct methods such as the euglycemic insulin clamp technique are more appropriate for assessment of insulin sensitivity (Donga *et al.*, 2015).

As discussed earlier, once the liver becomes fatty, both insulin sensitivity and clearance decrease. This implies that HOMA-IR might overestimate insulin resistance in individuals in whom insulin resistance is associated with a fatty liver (Kotronen, Vehkavaara, *et al.*, 2007).

2.3.3.1.2.1. Reference value for HOMA-IR

A recent joint European Practice guideline on NAFLD by the European Association for the Study of the Liver (EASL), the European Association for the Study of Diabetes (EASD) and the European Association for the Study of Obesity (EASO) (EASL *et al.*, 2016) stated: 'HOMA-IR provides a surrogate estimate of insulin resistance in persons without diabetes and can therefore be recommended provided proper reference values have been established'.

Reference intervals are usually defined according to the recommendations of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) (Solberg, 1987). A reference population is a group of reference individuals who are healthy people fulfilling pre-defined criteria. A reference value is measured from each reference individual by an appropriate method, and the statistical distribution of the reference values is then used to produce reference intervals. The reference interval is the area between and including both the lower and upper reference limits.

Reference intervals are usually defined in a population-based sample of age and gender-matched individuals as the mean ±2 standard deviation (SD) for variables with a Gaussian distribution, or as the central 95% reference interval (90% confidence interval [CI]) for variables that are non-normally distributed (Gräsbeck and Alström, 1981; Horn and Pesce, 2003). However, if the lower 2.5th percentile is not clinically relevant as in the case of HOMA-IR, a one-sided upper limit, the 95th percentile, may be used as the reference value (Horn and Pesce, 2003).

Regarding a reference value for HOMA-IR, the problem with this definition is that health then becomes dependent on the underlying population, as obesity is highly prevalent and perhaps the single most important cause of variation in insulin sensitivity. Thus for HOMA-IR, it would seem wiser to use reference values which are based on a study of only healthy subjects, although the definition of 'healthy' may also vary (Velho *et al.*, 2010). Controllable pre-analytical causes (the specimen collection technique, posture, fasting/postprandial, rest/exercise, drugs, stress, circadian variation) and non-controllable pre-analytical causes (age, gender, ethnicity) of variation should also be considered in defining reference value for HOMA-IR. Also, as discussed earlier, the inter-assay variation in insulin measurements must be taken into account when defining a reference value for HOMA-IR.

Three previous studies have been performed in 'healthy' and non-obese subjects (BMI <25 kg/m^2) comprising 161 Japanese subjects, 161 Italian subjects and 312 Brazilian subjects (Bonora *et al.*, 1998; Geloneze *et al.*, 2006; Nakai *et al.*, 2002). In the Japanese study, the 90th percentile of HOMA-IR was 1.7 (Nakai *et al.*, 2002). In the Italian study, the 80th percentile of HOMA-IR was 2.77 (Bonora *et al.*, 1998). In the Brazilian study, the 90th percentile was equally high, 2.71.

2.3.3.1.2.2. HOMA-IR in NAFLD

By definition, a number of studies have shown that subjects with 'Metabolic NAFLD' have higher HOMA-IR than subjects without NAFLD (Adams *et al.*, 2009; Angulo *et al.*, 2004; Brunt *et al.*, 2011; Chalasani *et al.*, 2003; Marchesini *et al.*, 1999; Targher *et al.*, 2006). HOMA-IR has a strong positive correlation with liver

fat content (Adiels *et al.*, 2007; Fedchuk *et al.*, 2014; Finucane *et al.*, 2013). Two earlier studies have defined a cut-off for HOMA-IR in the diagnosis of NAFLD. A study by Salgado et al. included 116 Brazilian subjects with NAFLD diagnosed by US (51%) or biopsy (49%) and in addition 88 healthy subjects (Salgado *et al.*, 2010). In receiver operating characteristics (ROC) curve analysis, a HOMA-IR of 2.0 with an AUROC of 0.84 (95% CI 0.78–0.90) was the best for distinguishing between NAFLD and non-NAFLD subjects. The sensitivity was 85% and specificity 83%. A study by Perez *et al.* included 263 Columbian men, of whom 27% had NAFLD as diagnosed by US (Perez *et al.*, 2011). The best cut-off for NAFLD was a HOMA-IR of 1.74 with an AUROC of 0.78, sensitivity of 74% and specificity of 73% (Perez *et al.*, 2011).

It is unknown how HOMA-IR relates to the normal amount of liver fat as defined in the DHS, and whether this definition reflects what normal liver fat is in other centers using ^{1}H-MRS. Moreover, the impact of PNPLA3 I148M variant on reference values for HOMA-IR has not been studied.

2.3.3.1.3. Phosphorylated IGFBP-1 (pIGFBP-1)

Insulin-like growth factor binding protein-1 (IGFBP-1) is one of six IGFBPs which regulate the bioavailability of insulin-like growth factor-1 (IGF-1) by binding to it (Firth and Baxter, 2002). In non-pregnant adults, the liver is the exclusive site of production of IGFBP-1 (Brismar *et al.*, 1994). Hepatocytes produce primarily phosphorylated IGFBP-1 (pIGFBP-1), which has a 6-fold higher affinity for IGF-1 than after dephosphorylation (Jones *et al.*, 1991). In circulation, pGFBP-1 is also the main form of IGFBP-1 (Jones *et al.*, 1991), but a small amount of IGFBP-1 that is not phosphorylated is also found in the circulation (Jones *et al.*, 1991).

Insulin is the main regulator of serum IGFBP-1 *in vivo* (Suikkari *et al.*, 1988). It acutely lowers IGFBP-1 concentrations (Suikkari *et al.*, 1988). Insulin sensitivity also regulates serum IGFBP-1 concentrations (Kotronen, Lewitt, *et al.*, 2008). Fasting IGFBP-1 is markedly lower in subjects with a fatty liver and hepatic insulin resistance than in subjects with fatty liver and normal hepatic insulin sensitivity (Kotronen, Lewitt, *et al.*, 2008).

Phosphorylated but no other forms of IGFPB-1 have been suggested to be associated with macrovascular complications of type 2 diabetes (Heald *et al.*, 2002). Serum pIGFBP-1 may also correlate better with cardiovascular risk factors than lesser-phosphorylated IGFBP-1 (Borai, Livingstone, Ghayour-Mobarhan, *et al.*, 2010). These data provide a rationale for measuring specifically pIGFBP-1 rather than IGFBP-1 as a marker of liver fat content and associated metabolic abnormalities.

As previously discussed, insulin assays are not well standardised and the concentrations may vary 2-fold when the same sample is measured using a different assay (Manley *et al.*, 2007). Earlier studies have used radioimmunoassay (RIA) to measure IGFBP-1. Phosphorylation status alters the antigenicity of IGFBP-1 (Westwood *et al.*, 1994) and therefore immunoassays may grossly underestimate changes in IGFBP-1 concentrations. However, an assay measuring pIGFBP-1 utilising antibodies and kits developed by one laboratory, Medix Biochemica (Kauniainen, Finland) (Rahkonen *et al.*, 2009; Riboni *et al.*, 2011), can avoid problems of standardisation between laboratories.

Previously, low fS-IGFBP-1 concentrations have been associated with NAFLD in studies comprising 142 Japanese subjects (Wasada *et al.*, 2008), 48 Italian women (Savastano *et al.*, 2011), and 49 African American and 77 Latino adolescents

(Alderete *et al.*, 2011). Liver fat content has been shown to correlate inversely with fS-IGFBP-1 in two studies comprising 48 subjects (Kotronen, Lewitt, *et al.*, 2008) and 113 subjects (Mofrad *et al.*, 2003). There is, however, no data on the relationship between fS-pIGFBP-1 and liver fat content or data examining whether the measurement of fS-pIGFBP-1 helps in the prediction of liver fat content compared to routinely available predictive markers.

2.3.3.2. Liver enzymes

The liver enzymes AST, ALT and gamma-glutamyltransferase (GGT) are routinely used surrogates of hepatocellular injury. ALT mostly originates from hepatocytes, while AST is also found in the heart and skeletal muscle, and GGT also in biliary epithelial cells and extrahepatic cells such as in the pancreas and renal tubules (Giannini, 2005). ALT is thus the most liver-specific of the enzymes, as increases in AST or GGT may also reflect extrahepatic pathology (Pratt and Kaplan, 2000). Increased serum GGT activity has also been used in clinical practice as a marker of excessive alcohol intake, especially if GGT is twice above normal in patients with an AST/ALT-ratio of at least 2:1 (Whitfield, 2008). In a Finnish population-based study including 2766 subjects, GGT concentrations were significantly higher in subjects with alcoholic fatty liver disease than with NAFLD, but no difference was observed in the AST/ALT-ratio between the two liver diseases (Kotronen *et al.*, 2010). NAFLD is the most common cause of elevation in these liver enzymes both in the United States (41% of increased ALT or 34% of increased AST due to NAFLD) (Lazo *et al.*, 2013), and in Finland (75% of increased ALT due to NAFLD) (Kotronen *et al.*, 2010).

Liver fat positively correlates with serum ALT, AST and GGT concentrations in men and women (Westerbacka *et al.*, 2004). The intercepts but not the slopes of the regression lines in the correlation between serum ALT and liver fat content differ between men and women (Westerbacka *et al.*, 2004). This is in line with a reference range of ALT lower for women than for men, probably reflecting the gender difference in liver size. Recently in Finland, many hospital districts adopted lower reference values for serum ALT, i.e., 50 U/l in men and 35 U/l in women, which were based on values from normal weight abstainers (Danielsson 2014; Niemelä and Danielsson, 2015).

Serum ALT is a poor marker of NAFLD. In a cohort of 222 subjects with biopsy-proven NAFLD, 23% of subjects had normal ALT levels, and 38% of patients with normal ALT levels had NASH or advanced fibrosis (Verma *et al.*, 2013). Despite normal ALT and AST levels, 56% of overweight or obese patients with type 2 diabetes had NAFLD (Portillo Sanchez *et al.*, 2015). According to a population-based study, up to 80% of subjects with NAFLD may remain undiagnosed if the diagnosis relies only on elevated liver enzymes (Browning *et al.*, 2004).

2.3.3.3. Scores

Various combinations of biochemical and anthropometric measurements have been proposed as helpful in identifying patients with NAFLD.

The Fatty Liver Index (FLI) was created on the basis of 216 Italian subjects with NAFLD and 280 without NAFLD diagnosed by US. The model included waist circumference, BMI, triglycerides and GGT (Bedogni *et al.*, 2006), and had an AUROC of 0.84 (95% CI 0.81–0.87). The sensitivity of a low cut-off to rule out NAFLD was 87%, and the specificity of a high cut-off to diagnose NAFLD was 86%. The FLI has been validated in 336 British subjects (^{1}H-MRS) (Cuthbertson *et al.*, 2014), 2652 Dutch subjects (US) (Koehler *et al.*, 2013), 3548 Chinese subjects (US)

(Xia, Yki-Järvinen, *et al.*, 2016), 324 French subjects (liver biopsy) (Fedchuk *et al.*, 2014) and 92 German subjects (^{1}H-MRS) (Kahl *et al.*, 2014). The AUROC of FLI was 0.79 (25–75% 0.74–0.84) in the British study, 0.81 (95% CI 0.79–0.82) in the Dutch study, 0.76 (95% CI 0.75–0.78) in the Chinese study and 0.83 (95% CI 0.72–0.91) in the French study and 0.72 (95% CI 0.59–0.85) in the German study.

The Hepatic Steatosis Index (HSI) was created on the basis of 5360 Korean subjects and validated in 5364 Korean subjects (Lee *et al.*, 2010). All subjects underwent US to diagnose NAFLD, and 2680 subjects of both cohorts had NAFLD. The HSI score included ALT/AST ratio, BMI, gender and diagnosis of diabetes. The AUROC for the model was 0.82 (95% CI 0.80–0.83). The sensitivity of the low-cut off for ruling out NAFLD was 90.4% and the specificity of the upper cut-off for diagnosing NAFLD was 92.4%. The HSI has been validated in 3548 Chinese subjects (US) (Xia, Yki-Järvinen, *et al.*, 2016), 324 French subjects (liver biopsy) (Fedchuk *et al.*, 2014) and 92 German subjects (^{1}H-MRS) (Kahl *et al.*, 2014). The AUROC of the HSI to diagnose NAFLD was 0.77 (95% CI 0.75–0.78) in the Chinese study, 0.81 (95% CI 0.72–0.88) in the French study, 0.79 (95% CI 0.68–0.90) in the German study.

Our group has previously developed a score for diagnosing NAFLD (the NAFLD Liver Fat Score) and an equation for estimating the amount of liver fat (NAFLD Liver Fat Equation) (Kotronen, Peltonen, *et al.*, 2009). These data were based on a study of 470 subjects who had undergone ^{1}H-MRS for measurement of liver fatcontent (313 subjects as a discovery and 157 subjects as a validation cohort) (Kotronen, Peltonen, *et al.*, 2009). Both scores included diagnosis of MetS and type 2 diabetes, fS-Insulin, AST and the AST/ALT ratio. The AUROC for the NAFLD Liver Fat Score was 0.88 (95% CI 0.84–0.92) with a sensitivity of the model was 86% and specificity of 71%. The correlation between measured liver fat content and liver fat content calculated from the NAFLD liver fat equation was $r=0.70$, $p<0.001$. The NAFLD Liver fat score has later been validated in 3548 Chinese subjects (US) (Xia, Yki-Järvinen, *et al.*, 2016), 324 French subjects (liver biopsy) (Fedchuk *et al.*, 2014) and 92 German subjects (^{1}H-MRS) (Kahl *et al.*, 2014). The AUROC of the NAFLD Liver Fat Score to correctly diagnose NAFLD was 0.78 (95% CI 0.72–0.77) in the Chinese study, 0.80 (0.69–0.88) in the French study, and 0.70 (0.53–0.87) in the German study. In the German study, the NAFLD Liver Fat Equation correlated with hepatic fat content ($r=0.42$, $p<0.001$) but the ratio of observed and predicted hepatic fat content ranged from 0.02 to 12.2 (Kahl *et al.*, 2014).

3 AIMS OF THE STUDY

The aims of the study were:

i. to determine whether SC adipocyte size is associated with liver fat content independent of other factors such as age, gender, obesity, adipose tissue distribution and *PNPLA3* genotype (study I);

ii. to systematically review definitions of normal liver fat using different diagnostic methods and how the definitions correspond to liver histology (study II);

iii. to systematically review whether there is a difference in insulin sensitivity between carriers and non-carriers of the PNPLA3 I148M variant, and carriers and non-carriers of the TM6SF2 E167K variant (study II);

iv. to determine reference values for HOMA-IR in two population-based cohorts and the inter-laboratory variation of HOMA-IR measurements in seven European laboratories (study III);

v. to define the HOMA-IR that best distinguishes between NAFLD and corresponds normal liver fat content, as quantified by ^{1}H-MRS (study III); and

vi. to determine whether pIGFBP-1 helps in the prediction of liver fat content in NAFLD compared to routinely available clinical and biochemical parameters (study IV).

4 SUBJECTS AND METHODS

4.1. SUBJECTS AND STUDY DESIGNS

For studies I, III (the 'Liver Fat Cohort') and IV, the study subjects were recruited for metabolic studies between May 2001 and October 2014 by newspaper advertisements, by contacting colleagues and occupational health services, or amongst subjects referred to the Department of Gastroenterology because of chronically elevated serum transaminase concentrations. Inclusion criteria were i) age 18 to 75 years, ii) no known acute or chronic disease other than obesity, type 2 diabetes, hypertension, hypercholesterolemia or NAFLD based on medical history, physical examination and standard laboratory tests (blood count, creatinine, electrolyte concentrations and electrocardiogram), iii) no pregnancy or lactation, iv) no evidence of pre-existing liver conditions other than NAFLD (e.g. autoimmune, viral or drug-induced liver disease) or a history of use of toxins or drugs associated with liver steatosis, antihypertensives possibly influencing glucose metabolism or TZDs, v) no excessive use of alcohol (over 20 g/day for women and 30 g/day for men). Study physicians assessed alcohol intake by using the same questionnaire addressing the quantity of different alcoholic drinks consumed during an average week. The study protocols were approved by the Ethics committee of Helsinki University Central Hospital and written informed consent was obtained from all study subjects.

4.1.1. Adipocyte size in NAFLD

Non-diabetic study subjects (n=119) participated in a metabolic study. The subjects were studied after an overnight fast. Body composition was measured as detailed below in section 4.2.4. Blood was drawn for measurement of complete blood count, serum total cholesterol, HDL and LDL cholesterol, triglyceride and plasma glucose concentration, and glycated haemoglobin A_{1c} (HbA_{1c}), serum insulin and C-peptide, ALT and AST concentrations. Blood samples were also obtained for genotyping *PNPLA3* at rs738409 as described below in section 4.2.6. Thereafter, a biopsy of abdominal subcutaneous adipose tissue was taken under local anaesthesia with 1% lidocaine by a needle aspiration (Yki-Järvinen *et al.*, 1986). In addition, on another occasion liver fat was measured with ^{1}H-MRS, and abdominal SC and IA adipose tissue volumes were determined with MRI. Data on these subjects has been reported before (Kotronen, Peltonen, *et al.*, 2009).

4.1.2. Definition of normal liver fat and insulin sensitivity in 'Genetic NAFLD'

A systematic review was performed on two topics: i) definitions of normal liver fat using ^{1}H-MRS, MRI, US and CT, and ii) comparison of insulin sensitivity in carriers and non-carriers of the PNPLA3 I148M variant, and the carriers and non-carriers of the TM6SF2 E162K variant.

4.1.3. Reference values for HOMA-IR and an optimal cut-off for NAFLD

For determination of reference values for HOMA-IR, we studied non-pregnant adults in two population-based cohorts, the National FINRISK 2007 study conducted by the National Institute for Health and Welfare in Finland (Vartiainen *et al.*, 2010) and the Programme for Prevention of Type 2 Diabetes in Finland (FIN-D2D) (Saaristo *et al.*, 2007). Health was defined using the same criteria as in the population-based DHS (Szczepaniak *et al.*, 2005): i) alcohol use less than 30 g/day in men and less than 20 g/day in women, ii) non-diabetic based on history and

normal fP-glucose (<6.1 mmol/l), iii) non-obese (BMI <25 kg/m^2), and iv) no clinical or biochemical evidence of other liver disease or metabolic syndrome as defined by history and biochemical examinations (*vide infra*).

FINRISK/DILGOM Study. The participants took part in two phases of the National FINRISK 2007 Study conducted by the National Institute for Health and Welfare in Finland (Vartiainen *et al.*, 2010). An independent random sample of 10 000 men and women aged 25 to 74 years was drawn from the national population register in five geographical areas at the end of 2006. The sample was stratified by sex, 10-year age category and area. The first phase took place between January and March 2007, and included a health examination in local health centres or other survey sites by specially trained nurses. At this visit, weight, height, waist circumference, hip circumference and blood pressure were measured and blood was drawn. Health questionnaires were mailed together with an invitation to the health examination. These addressed socio-demographic factors, health behaviour and medical history and included a detailed questionnaire regarding weekly (past week) and yearly (past year) alcohol consumption. A total of 6258 participants who took part in the first phase of the survey were invited to a more detailed examination of the dietary, lifestyle and genetic determinants of obesity and the metabolic syndrome from April to June 2007. This second phase comprised the study of the Dietary Lifestyle and Genetic Determinants of the Development of Obesity and Metabolic syndrome (the DILGOM Study) (Saaristo *et al.*, 2007). It also included anthropometric measurements and collection of blood samples to determine concentrations of fasting plasma glucose, serum insulin and lipids. The response rate to the second phase was 80%, and thus the cohort included 5024 subjects. The protocol was approved by the Ethics Committee of Helsinki and Uusimaa Hospital District. Written informed consent was obtained from all the participants for both field research phases.

FIN-D2D Study. The Programme for Prevention of Type 2 Diabetes in Finland (FIN-D2D) was carried out in three hospital districts in Finland between October and December 2007 (Männistö *et al.*, 2014). A random sample of 4500 subjects aged 45 to 74 years (stratified according to gender, 10-year age groups and the three geographical areas) was selected from the National Population Register. The overall participation rate was 64%. Nineteen subjects were excluded from analyses due to missing anthropometric data. The total number of individuals included was thus 2849 representing 63% of the random sample. The subjects were invited by mail to a clinical examination. They also received a self-administered questionnaire on medical history and health behaviour, which included a detailed questionnaire regarding weekly (past week) and yearly (past year) alcohol consumption. The questionnaire was filled in at home and brought to the health examination. A trained nurse measured body composition and drew blood for measurement of fasting plasma glucose, serum insulin, lipids, HbA_{1c} and liver enzyme concentrations. All samples were collected after an overnight fast. The study protocol was approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa, and all participants gave their written and informed consent.

Liver Fat Cohort. 368 non-diabetic subjects participated in a metabolic study. The subjects were studied after an overnight fast. Body composition was measured as detailed later in section 4.2.4. Blood was drawn for measurement of complete blood count, serum total, HDL and LDL cholesterol, triglyceride and plasma glucose concentration, and HbA_{1c}, serum insulin and C-peptide, and ALT,

AST and GGT concentrations. Blood samples were also taken for genotyping *PNPLA3* at rs738409 as described below in section 4.2.6. On another occasion, liver fat was measured with ^{1}H-MRS. Data on 273 subjects has been reported before (Kotronen, Peltonen, *et al.*, 2009).

Inter-laboratory variation study. Ten non-diabetic volunteers covering a wide range of insulin sensitivity were recruited in May 2016. The subjects were healthy based on medical history, physical examination and standard laboratory tests but 8 of them had BMI ≥25 kg/m^2. Blood was drawn in Helsinki after a 12-h fast for measurement of fasting insulin, glucose, HDL, LDL and total cholesterol, triglycerides, AST, ALT, GGT, ferritin and albumin. Measurements of biochemical markers other than insulin and glucose were performed for comparative purposes to estimate their inter-assay CVs. In Helsinki, the samples were analysed immediately. Another set of samples were instantly frozen to -80 °C and then thawed and assayed the same day in Helsinki to study the effect of freezing. To study the effect of time, a third set of samples were instantly frozen to -80 °C, and thawed and assayed after 2 weeks in Helsinki. At this same time point, six additional sets of samples, which had been shipped to the participating centres on dry ice, were thawed and assayed at Newcastle University (Newcastle, UK), Johannes Gutenberg University Mainz (Mainz, Germany), the Institute of Clinical Physiology (Clinical and Research laboratories) (Pisa, Italy), the University of Torino (Torino, Italy), and the Institute of Cardiometabolism and Nutrition (Paris, France). The study protocol was approved by the Ethics Committee of Helsinki University Central Hospital and was carried out in accordance with the Declaration of Helsinki. Each participant provided written informed consent.

4.1.4. pIGFBP-1 in NAFLD

378 subjects participated in metabolic studies. The subjects were studied after an overnight fast. Body composition was measured as detailed later in section 4.2.4. Blood was drawn for measurement of plasma glucose concentration, serum total, HDL and LDL cholesterol, triglycerides, HbA_{1c}, insulin, C-peptide, pIGFBP-1, ALT, and AST concentrations. Blood samples were also taken for genotyping *PNPLA3* at rs738409 as detailed later in section 4.2.6. On another occasion, liver fat was measured using ^{1}H-MRS. Data on the subjects had been reported previously (Kotronen, Peltonen, *et al.*, 2009).

4.2. METHODS

4.2.1. Systematic review (II)

A systematic search using PubMed and Ovid MedLine was performed in October 2015 on two topics.

i) To identify definitions of normal liver fat, the following search terms and their combinations were used: 'normal liver fat', combined with 'liver histology', 'liver biopsy', 'liver H-MRS', 'liver MRI', 'liver MRI-PDFF', 'liver CT' and 'liver ultrasound'. Out of the 526 hits, 33 studies matched the criteria and assessed normal liver fat content or comparison of liver fat content using different techniques and were hence included in the review.

ii) The association between insulin resistance and NAFLD due to PNPLA3 I148M or TM6SF2 E167K variant was assessed by performing a systematic search using the following search terms: 'PNPLA3' or 'TM6SF2' combined with 'insulin resistance', 'euglycemic [hyperinsulinemic] clamp', 'fasting glucose', 'fasting insulin', 'HOMA-IR' and 'oral glucose tolerance test [OGTT]'. From the 124 studies matching the search terms, 22 studies included data on liver fat

content between carriers and non-carriers of either PNPLA3 I148M or TM6SF2 E167K variant and on insulin sensitivity assessed by the aforementioned methods, and were thus included.

4.2.2. Liver fat content using ^{1}H-MRS (I, III, IV)

Three generations of 1.5 Tesla clinical scanners, Vision, Sonata and Avanto, manufactured by Siemens Healthcare Diagnostics (Erlangen, Germany) were used in the measurements and thus the intensity differences arising from various acquisition parameters and localization techniques had to be normalized. T1-weighted localisation images were collected using a standard ^{1}H body coil. An 8 to 27 cm^3 ^{1}H-MRS voxel was carefully positioned in the right lobe of the liver avoiding subcutaneous fat, large vessels, bile ducts and gall bladder. Localization was carried out using the STEAM sequence with TE (echo time)/TM (mixing time)/TR (repetition time) of 20/30/3000 ms and 32 acquisitions for Vision measurements and PRESS sequence with TE/TR of 30/3000 ms and 16 acquisitions for Sonata and Avanto measurements. Subjects were breathing normally during the data collection. All spectra were analysed with the MRUI/jMRUI software using VARPRO/AMARES (www.mrui.uab.es/mrui/). The intensities of the peaks resonating from the protons of water, and protons of methylene [$(CH_2)n_{-2}$] groups in the fatty acid chains were determined using lineshape fitting with prior knowledge. Signal intensities were corrected for T1 and T2 relaxation using the equation $I_m=I_0$ exp(−TE/T2)*[1−exp(−(TR−TM−0.5TE)/T1)]*exp(−TM/T1) for Vision data and the equation $I_m=I_0$ exp(−TE/T2) for Sonata/Avanto data. T1 of 600 ms (Stanisz GJ *et al.*, 2005) and 300 ms (Graham *et al.*, 1999) and experimentally determined T2 of 46 ms and 58 ms were used for water and fat, respectively. Liver fat content was expressed as a ratio of signal from methylene group to total signal of methylene and water. Liver fat content was converted from signal ratio to a weight fraction, applying method validated by Longo *et al.* (Longo *et al.*, 1995) and Szczepaniak *et al.* (Szczepaniak *et al.*, 2005). The following experimentally determined factors were used: i) the ratio of the number of lipid protons in the fitted $(CH2)_{n-2}$ signal to the total number of lipid protons is 0.6332 (Szczepaniak *et al.*, 1999); ii) proton densities of fat and water are 111 and 111 mol/l, respectively; iii) 1 g liver tissue contains 711 mg water; iv) densities of the liver tissue, fat in the liver, and water are 1.051 g/ml, 0.900 g/ml, and 1.000 g/ml; respectively. All spectra were analysed by a physicist who was unaware of the clinical data. NAFLD was defined as liver fat ≥55.6 mg triglyceride/g liver tissue or >5.56% of liver tissue weight as in the DHS (Szczepaniak *et al.*, 2005).

4.2.3. Adipocyte size and number (I)

SC adipocyte size was determined using the collagenase digestion method (Rodbell, 1964). In brief, adipose tissue from a needle aspiration biopsy was transferred into a 2 ml plastic vial that contained collagenase buffer (100 mg of collagenase [C-6885, Type II, Sigma-Aldrich, St. Louis, MO], 0.1 ml of a 550 nM glucose solution and 35 ml 10% albumin in HEPES buffer in a total volume of 100 ml). The sample was incubated in a water bath at 37°C for one hour. The adipocytes that floated on the surface were then transferred into counting vials using a disposable syringe (Bürker, Marienfeld-Superior, Lauda-Könighofen, Germany). Adipocyte cell sizes were analysed using a light microscope (Leica DM750 Led, Wetzlar, Germany) equipped with a caliper scale, and the 'adipocyte size' was defined as the mean size of 100 cells. The total number of adipocytes in the body was calculated as follows: i) total body fat mass was derived from body fat percentage and total body weight; ii) total fat volume was calculated as fat mass (kg) divided by its density,

0.9196 kg/m^3 (Abate *et al.*, 1996); iii) adipocytes were assumed to be spherical, and their volume calculated with the formula for the volume of sphere ($V=4/3\ \pi\ r^3$); and iv) the total number of adipocytes was derived by dividing total volume of whole body fat by the volume of a single adipocyte.

4.2.4. Body composition (I, III, IV)

During the metabolic study visit, body weight was recorded to the nearest 0.1 kg using a calibrated digital scale (Soehnle, Monilaite-Dayton, Finland) with the subject barefoot and wearing light indoor clothing. Height was recorded to the nearest 0.5 cm using a non-stretchable tape. BMI was defined as [weight (kg)]/[height (m^2)]. Waist circumference was measured midway between superior iliac spine and the lower rib margin, and hip circumference at the level of the greater trochanters. Fat free mass and body fat percentage were determined using bioelectric impedance analysis (BioElectrical Impedance Analyzer, model #BIA-101A;RJL Systems, Detroit, MI).

4.2.5. IA and SC adipose tissue volume (I)

The MRI studies were carried out using the same scanners as detailed 4.2.2. A series of T1-weighted transaxial images were acquired from a region extending from 8 cm above to 8 cm below the L4/5 intervertebral disc (16 slices, field of view 375 x 500 mm^2, slice thickness 10 mm, breath-hold repetition time 138.9 ms, echo time 4.1 ms), as previously described (Ryysy *et al.*, 2000). For the data collected with Siemens Vision, the IA and SC fat areas were calculated in a blinded fashion using an image analysis program (Alice 3.0, Parexel, Waltham, MA). The data collected with Siemens Sonata and Avanto were analysed using SliceOmatic version 4.3 (TomoVision, Magog, Canada) segmentation software. The areas of the SC and IA adipose tissue were measured for each slice using a region-growing routine. A histogram of pixel intensity in the IA region was displayed, and the intensity corresponding to the nadir between the lean and fat peaks was used as a cut-off point. The IA adipose tissue was defined as the area of pixels in the intra-abdominal region above this cut-off point. For calculation of the SC adipose tissue area, a region of interest was first manually drawn at the demarcation of the SC adipose tissue and IA adipose tissue.

4.2.6. Genotyping of *PNPLA3* genotype at rs738409 (I, III, IV)

Studies I, the Liver Fat Cohort in III and IV. Approximately 10 ng of genomic DNA extracted from whole blood was used for genotyping by the TaqMan PCR method (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Post-PCR allelic discrimination was carried out measuring allele-specific fluorescence on an ABI Prism Sequence Detection System ABI 7900HT (Applied Biosystems). As this assay is designed for the reverse strand, the G allele corresponds to the 148Met phenotype while the gene is transcribed from the forward strand. The success rate for genotyping was >95%. The genotype was in the Hardy-Weinberg equilibrium.

FINRISK/DILGOM cohort (III). The *PNPLA3* genotype was determined from 1000G imputed GWAS data consisting of three subsets genotyped using Illumina Human Core Exome, Illumina Omni Express and Illumina 610K (Illumina, San Diego, CA).

FIN-D2D cohort (III). Genomic DNA was extracted from whole blood using automated Chemagen DNA extraction equipment (PerkinElmer, Waltham, MA), or a QIAamp DNA Blood Maxi Kit (QIAGEN GmbH, Hilden, Germany) following the protocol of the kit with slight modifications. Genotyping was performed using a TaqMan assay (Applied Biosystems, Paisley, UK).

4.2.7. S-pIGFBP-1 (IV)

Serum pIGFBP-1 concentrations were determined with an immunoenzymatic assay (IEMA) with monoclonal antibodies (Medix Biochemica, Kauniainen, Finland) according to the manufacturer's instructions (Nuutila M *et al.*, 1999). This assay uses a monoclonal antibody specific to human pIGFBP-1, which is immobilised on microwell plates, and a monoclonal antibody specific to IGFBP-1, which is bound to the microwells and conjugated with horse-radish peroxidase. The enzymatic reaction is proportional to the amount of pIGFBP-1 in the sample. The intra-assay CV was 2.7% to 7.8% and inter-assay CV 3.9% to 10%. Each sample was assayed in duplicate and the mean value was used. The detection limit of the assay was 0.3 μg/l and the measuring range 1 to 200 μg/l. No cross-reactivity with other IGFBPs was detected. All sera were analysed after storage at -80°C until analysis.

fS-IGFBP-1 was measured by RIA (Kotronen, Lewitt, *et al.*, 2008) in 23 samples that had been stored for 5 years. These samples were re-assayed with the IEMA for pIGFBP-1. The mean concentrations were 18±2 μg/l with the RIA and 57±8 μg/l with the IEMA ($p<0.001$ for the difference). The concentrations correlated positively ($r=0.64$, $p<0.001$).

4.2.8. Biochemical analysis (I, III, IV)

I, the Liver Fat Cohort in III and IV: Plasma glucose was measured using the hexokinase method, serum ALT, AST and GGT activities were according to the recommendations by the European Committee for Clinical Laboratory Standards, and serum triglyceride, total, LDL and HDL cholesterol concentrations in an automatic analyser (Roche Diagnostics Hitachi 917, Hitachi Ltd., Tokyo, Japan). Serum insulin and C-peptide concentrations were measured using time-resolved fluoroimmunoassay with Auto-DELFIA kits (Wallac, Turku, Finland). HbA_{1c} was measured using high-pressure liquid chromatography using a fully automated system (Bio-Rad, Richmond, CA, US).

FINRISK/DILGOM and FIN-D2D: Biochemical assays were performed in the Laboratory of Analytical Biochemistry of the Institute of Health and Welfare (Helsinki, Finland) using Architect ci8200 analyser (Abbott Laboratories, Abbott Park, IL, US). Plasma glucose was determined using the hexokinase method and serum insulin using a chemiluminescent microparticle immunoassay. Serum total and HDL cholesterol, and triglyceride concentrations were measured with enzymatic methods. The concentration of LDL cholesterol was calculated using the Friedewald formula (Friedewald *et al.*, 1972). Total cholesterol was measured with the CHOD-PAP-assay. Samples were stored at -80 °C before analysis. In the *FIN-D2D* study, HbA_{1c} was measured by an immunoturbidimetric method and serum ALT, AST, and GGT concentrations by using photometric IFCC methods.

Inter-laboratory study: Methods used in the inter-laboratory study are presented in Table 1.

HOMA-IR was calculated as: [fP-glucose (mmol/l) x fS-insulin (mU/l)]/22.5.

4.2.9. Statistical analysis (I, III and IV)

The distribution of continuous variables was tested for normality using the Kolmogorov-Smirnov test. Non-normally distributed data were subjected to logarithmic transformation. To compare characteristics between groups, the unpaired *t*-test and the Mann–Whitney's U test were used for continuous variables, and Fisher's exact test and χ^2 test for categorical variables when appropriate.

Table 1. Methods used in the inter-laboratory study (III) (company and method presented)

	Helsinki	Paris	Torino	Pisa (Research)	Pisa (Clinical)	Mainz	Newcastle
Insulin	DiaSorin, Liaison XL	DiaSorin, Liaison XL	Siemens, Immulite	Merck, Milliplex	Abbott, Architect i1000	Abbott, Architect i2000	Grifols, Triturus
	CLIA	CLIA	ICMA	ELISA	CMIA	CMIA	ELISA
Glucose	Abbott, Architect c16000	Roche, Modular P800	Roche Cobas 8000	Beckmann, Au400	Beckmann, DXC600	Abbott, Architect c8000 and c16000	Roche, Cobas 8000 C702
	Hexokinase	Hexokinase	Hexokinase	Hexokinase	Hexokinase	Hexokinase	Hexokinase
ALT	"	"	"	"	"	"	"
	Photometric IFCC	Photometric IFCC	Photometric IFCC	Photometric IFCC	Photometric IFCC	NADH Oxidation	Photometric IFCC
AST	"	"	"	"	"	"	"
	"	"	"	"	"	"	"
GGT	"	Enzymatic colorimetric	Szasz	Szasz	"	Photometric IFCC	Enzymatic colorimetric
	"						
Total cholesterol	"						
	CHOD-PAP	CHOD-PAP	CHOD-PAP	CHOD-PAP	CHOD-PAP	CHOD-PAP	CHOD-PAP
HDL cholesterol	"	"	"	"	"	"	"
	"	"	"	"	"	"	"
Triglycerides	"	"	"	"	"	"	"
	GPO-PAP	GPO-PAP	GPO-PAP	GPO-PAP	GPO-PAP	GPO-PAP	GPO-PAP
LDL cholesterol	"						
	Photometric, enzymatic	Friedewald formula	Friedewald formula	Friedewald formula	Friedewald formula	Friedewald formula	Friedewald formula
Albumin	"	Roche, Cobas 6000	Roche, Cobas 8000	-	Beckmann, DXC600	Abbott, Architect c8000 and c16000	"
	BCP	ITA	BCG	-	BCG	BCG	Colorimetric
Ferritin	Abbott, Architect i2000SR	"	"	-	DiaSorin, Liaison XL	Abbott, Architect i2000	"
	CMIA	"	CMIA	-	CLIA	CMIA	

", the same company/method as the one above.

Companies: Abbott Laboratories (Abbott, Lake Bluff, Illinois, USA), Beckmann Coulter (Brea, CA, USA), DiaSorin (Saluggia, Italy), Grifols (Sant Cugat del Valles, Barcelona), Konelab by Thermo Scientific (Waltham, Massachusetts, USA), Merck (Darmstadt, Germany), Roche Diagnostics (Basel, Switzerland), Siemens (Erlangen, Germany).

Abbreviations: BCG, bromocrescol green; BCP, bromocrescol purple; CMIA, chemiluminescent microparticle immunoassay; CLIA, chemiluminescence immunoassay; ECLIA, electrochemiluminescence immunoassay; CHOD-PAP and GPO-PAP, colometric enzymatic assays; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine; ITA, immunoturbidimetric assay.

Pearson's correlation coefficient or Spearman's rank correlation coefficient were used for univariate analysis (I, III, IV). Multiple linear regression analyses were performed to identify independent determinants of liver fat (I, III, IV). Bootstrap randomisation was used to randomise the subjects into discovery (2/3 of subjects) and validation (1/3 of subjects) cohorts, and all subjects were used as a second validation cohort (III, IV). P-values <0.05 were considered statistically significant. Normally distributed data are shown as mean ± SD, whereas non-normally distributed are shown as median (25–75th percentile). The calculations were performed using GraphPad Prism (versions 4.03 and 6.00, GraphPad Software Inc., San Diego, CA), SPSS Statistics (versions 17.0 and 21.0, SPSS Inc., Chicago, IL), Microsoft Office Excel (versions 2007 and 2011, Microsoft, Redmond, WA) and the R Project (version 3.1.1, www.r-project.org, Vienna, Austria).

Adipocyte size in NAFLD (I)

Spearman correlation coefficient was used to determine correlates of liver fat content. Partial correlation analysis was used to control for known covariates of liver fat. One-way ANOVA was used to compare liver fat content between the *PNPLA3* genotypes. Multiple linear regression analysis was used to search for independent predictors of liver fat content. Predictive models were compared using the *F*-test based on the residual sum of squares adjusted for the total number of variables in each model.

Reference value for HOMA-IR (III)

HOMA-IR was not normally distributed and therefore the 95th percentile (90% CI) rather than the mean + 2 SD was used to determine the upper reference value for HOMA-IR (Horn and Pesce, 2003). To evaluate the effect of BMI and gender, values of HOMA-IRs were subjected to $\log_2$ transformation and further adjusted in a generalised linear model by using age and BMI as covariates. The 95th percentile was also defined for S-ALT in the FIN-D2D cohort.

Determination of an optimal HOMA-IR cut-off value for NAFLD (III)

The HOMA-IR value corresponding to the normal liver fat based on the DHS (liver fat=5.56%) was calculated using linear regression analysis. We tested whether the slopes and intercepts in linear regression analysis differed between men and women, and carriers and non-carriers of the PNPLA3 I148M variant. The 95th percentile of liver fat in the healthy subjects of the Liver Fat Cohort was used to define normal liver fat content as in the DHS (Szczepaniak *et al.*, 2005).

The discovery group was used to determine the ROC curve for HOMA-IR and AUROC (95% CI). The Youden Index, which indicates the point of optimal sensitivity and specificity (Greiner *et al.*, 2000), was calculated to define the optimal cut-off of HOMA-IR to identify subjects with or without NAFLD. The validation group and all subjects were used for validation. For additional validation, we generated 1000 random sets of samples and used the bootstrap method to validate the model in the sample sets. The AUROC of each set was estimated, and the average of these estimates provided the overall prediction accuracy of the model. Power analysis was conducted to estimate the appropriate sample size for correlation analysis and ROC analysis. To detect a correlation coefficient of 0.2 between HOMA-IR and liver fat content with a power of 0.8, a sample size of at least 193 was required. By setting the ratio of sample sizes between negative and positive groups at 2, at least 23 cases and 46 control participants were needed to reach a statistical power of 0.8 to detect the minimum AUROC of 0.7.

The inter-laboratory variation of HOMA-IR and other analytes (III)

The inter-laboratory CVs of fasting insulin, glucose and HOMA-IR, lipids, liver enzymes, ferritin and albumin between laboratories were calculated as a the ratio of SD to mean. Linear regression analyses were performed to compare insulin, glucose and HOMA-IR measurements in Helsinki to those in other centres. Equations from these linear regression curves were used to define the HOMA-IR in each centre corresponding to the upper reference limit for HOMA-IR in Helsinki.

fS-pIGFBP-1 in NAFLD (IV)

Linear regression and Random Forest prediction models were used to estimate the liver fat content. For both models, variables that significantly correlated with liver fat in univariate analyses in the discovery group were used. One variable from each group of variables reflecting the same biological phenomenon (body composition, liver enzymes, glycaemia, insulinemia and lipids) was entered into the models to avoid multi-collinearity. Using a sample size of 378 subjects, a power of 0.8 and a P-value of 0.05, a linear correlation coefficient of 0.144 or over can be detected.

Multiple linear regression analysis was used to create an equation to estimate liver fat content. The final variables for this were derived from a backward stepwise regression method based on Akaike Information Criteria (AIC). The model was evaluated using adjusted coefficient of determination (R^2). Predictive models were compared using the *F*-test based on the residual sum of squares adjusted for the total number of variables in each model.

To compare the accuracy of the equation created using multiple linear regression with FLI (Bedogni *et al.*, 2006) and HSI (Lee *et al.*, 2010), their respective reference values were used and for the created equation the 5.56% reference value as a cut-off for NAFLD (Szczepaniak *et al.*, 2005). The ROC curve was determined to calculate AUROC for each predictive model, and the AUROCs were compared using the DeLong method (DeLong *et al.*, 1988).

In the Random Forest modelling, the optimal number of variables on each tree was defined based on the estimation of out-of-Bag error. By using the predictors described above, 500 regression trees were trained in the discovery group. The predictability of each variable was estimated by cross-validating its relationship with the outcome in the validation group and all subjects. A variable importance plot based on the importance score summarised the importance of each predictor. Correlation coefficients were compared statistically using the Fisher r-to-z transformation (Fisher, 1915).

5 RESULTS

5.1. SUBJECT CHARACTERISTICS (I, III–IV)

Physical and biochemical parameters of the study subjects in studies I, III and IV are shown in Table 2. 60% to 70% of study subjects were women and the median age was between 42 and 55 years. Apart from the FINRISK/DILGOM and FIN-D2D cohorts, which were by definition non-obese, the study subjects were mostly overweight or obese. 35% to 46% of the subjects had NAFLD (studies I, the Liver Fat Cohort in III and IV). 51% to 60% did not carry the PNPLA3 I148M gene variant.

5.2. ADIPOCYTE SIZE IN NAFLD (I)

In this study, women had significantly higher HDL cholesterol concentration, abdominal SC adipose tissue volume and adipocyte number than men ($p<0.0005$), whereas men had a significantly higher waist-to-hip ratio and fS-ALT concentration ($p<0.005$) (I, Table 1).

5.2.1. Adipocyte size and liver fat

The median (25–75%) adipocyte size was 112 (96.2–120) μm and adipocyte number 4.07 (3.29–5.28) x 10^9. Adipocyte size correlated significantly with liver fat (all subjects $\rho=0.50$, $p<0.0001$; for men $\rho=0.67$, $p<0.0001$; and for women $\rho=0.41$, $p<0.0001$). Other parameters that correlated with liver fat in univariate analysis included age ($\rho=0.30$, $p=0.001$), measures of obesity, fS-insulin ($\rho=0.59$, $p<0.0001$), fS-C-peptide ($\rho=0.54$, $p<0.0001$), measures of glycaemia, fP-triglycerides ($\rho=0.44$, $p<0.0001$), liver enzymes, and *PNPLA3* genotype (I, Table 1). Adipocyte number did not correlate significantly with liver fat content ($p=0.18$).

The relationship between adipocyte size and liver fat remained significant after adjustment for the factors known to regulate liver fat content, i.e., age, gender, BMI, *PNPLA3* genotype and the IA/SC adipose tissue volume ratio (adjusted correlation coefficient for all subjects $\rho=0.44$, $p<0.0001$), Fig. 1.

To determine whether adipocyte size is an independent determinant of variation in liver fat, results of the univariate analysis and physiologically plausible regulators of liver fat (adipocyte size, body fat distribution, BMI, *PNPLA3* genotype, age, gender) were subjected to multiple linear regression analysis. A model including age, BMI, IA/SC adipose tissue ratio, *PNPLA3* genotype and adipocyte size accounted for 53% of the variation in liver fat (Table 3), and exclusion of adipocyte size reduced the explanatory power of the model significantly to 42% ($p<0.0001$). Gender did not remain significant ($p=0.07$).

Table 2. Characteristics of subjects (studies I, III-IV)

	Study I	Study III FINRISK/DILGOM	Study III FIN-D2D	Study III Liver Fat Cohort	Study III Inter-laboratory	Study IV
	n=119	n=1167	n=549	n=368	n=10	n=378
Age (years)	39 (26-53)	44 (35-56)	55 (50-62)	42 (28-52)	42.8±5.2	43 (30-54)
Gender (%, women/men)	70/30	68/32	67/33	60/40	70/30	62/38
BMI (kg/m^2)	30.0±5.7	22.7 (21.4-24.0)	22.9 (21.6-24.1)	28.8 (24.5-33.2)	30.1±2.3	32.0 (28.1-37.9)
Waist-to-hip ratio	0.91 (0.86-0.97)	-	-	0.91 (0.85-0.97)	0.91±0.10	0.94 (0.88-1.02)
fS-HDL cholesterol (mmol/l)	1.4 (1.2-1.7)	1.5 (1.4-1.8)	1.6 (1.4-1.8)	1.4 (1.2-1.7)	1.7±0.1	1.3 (1.0-1.6)
fS-LDL cholesterol (mmol/l)	2.9±0.9	3.0 (2.4-3.5)	3.3 (2.8-3.9)	3.0 (2.4-3.6)	2.6±0.3	2.6 (2.1-3.4)
fS-Triglycerides (mmol/l)	1.2 (0.8-1.6)	0.8 (0.6-1.0)	0.9 (0.7-1.1)	1.1 (0.8-1.6)	1.4±0.3	1.3 (1.0-1.9)
fP-Glucose (mmol/l)	5.4 (5.0-5.8)	5.5 (5.2-5.7)	5.6 (5.4-5.8)	5.4 (5.0-5.8)	5.8±0.2	5.7 (5.2-6.5)
fS-Insulin (mU/l)	7.1 (4.6-11)	4.1 (3.3-5.3)	4.5 (3.4-5.6)	6.7 (3.7-11.0)	8.8±1.5	9.3 (6.0-15.0)
fS-C-peptide (nmol/l)	0.8±0.4	-	-	0.68 (0.49-0.98)	-	0.94 (0.58-1.23)
HOMA-IR	-	1.02 (0.8-1.3)	1.1 (0.8-1.4)	1.6 (0.8-2.7)	2.3±0.5	-
HbA_{1c} (%)	5.5 (5.3-5.7)	-	5.1 (4.9-5.3)	5.5 (5.3-5.7)	-	5.7 (5.4-6.2)
fS-ALT (U/l)	27 (19-51)	-	18 (15-23)	25 (18-39)	27±4	32 (21-51)
fS-AST (U/l)	28 (23-38)	-	22 (18-26)	26 (22-32)	34±6	28 (23-40)
fS-GGT (U/l)	24 (15-46)	-	19 (14-28)	23 (15-40)	23±6	-
fP-Albumin (g/l)	-	-	-	-	40±1	-
fP-Ferritin (µg/l)	-	-	-	-	53.2±12.5	-
Liver fat (%)	3.4 (1.1-9.2)	-	-	2.7 (0.9-8.6)	-	4.9 (1.0-12.4)
NAFLD (%)	40%	-	-	35%	-	46%
PNPLA3 genotype (%, I148II or. I148IM/MM)	51/49%	59/41%	60/40%	60/40%	-	60/40%

data shown as mean±SD or median (25-75%)

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; fP, fasting plasma; fS, fasting serum; HbA_{1c}, glycated haemoglobin A_{1c}; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; PNPLA3, patatin-like phospholipase domain containing 3

Figure 1.

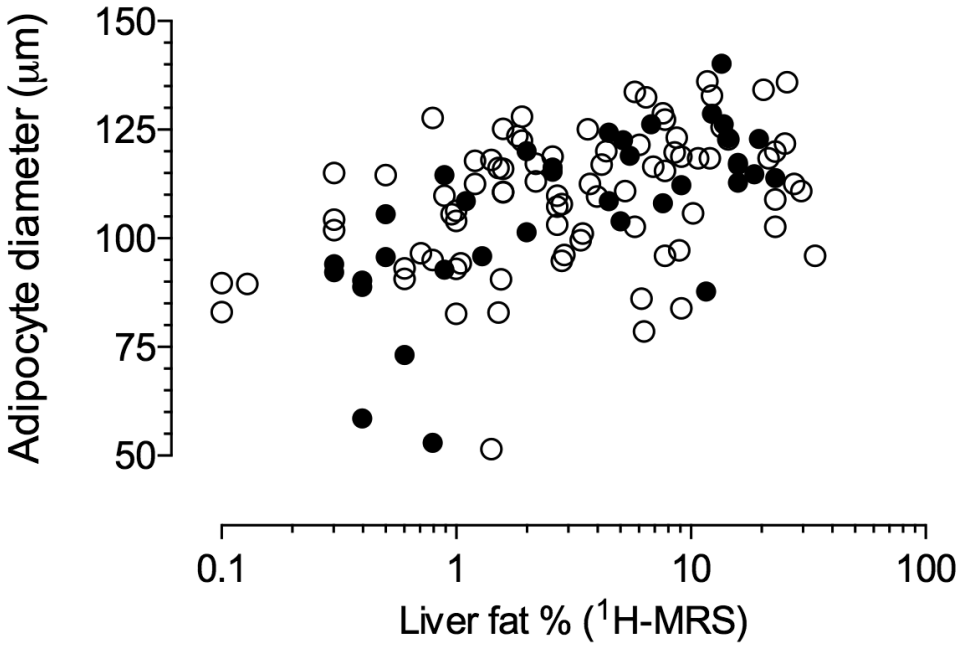

Figure 1. Correlation between liver fat content and adipocyte diameter controlled with age, gender, BMI, *PNPLA3* genotype, IA/SC adipose tissue volume ratio (ρ=0.44, p<0.0001). Full circles, men; open circles, women. Adapted from Petäjä EM *et al*. *Obesity* 2013; 21: 1174–9 and reproduced with the permission of John Wiley and Sons.

Table 3. Determinants of liver fat in multiple linear regression analysis (R^2=53%, adjusted R^2=51%)

	Unstandardised coefficient β	Standard error	*P* value
Age (log_{10})	-0.74	0.32	0.022
BMI	0.033	0.008	<0.0001
IA/SC adipose tissue volume (log_{10})	0.78	0.18	<0.0001
PNPLA3 genotype*	0.29	0.057	<0.0001
Adipocyte size (log_{10})	3.35	0.67	<0.0001

** 1=I148II, 2=I148IM, 3=I148MM. BMI, body mass index; IA, intra-abdominal; PNPLA3, patatin-like phospholipase domain containing 3; SC, subcutaneous. Adapted from Petäjä EM et al. Obesity 2013; 21: 1174-9 and reproduced with the permission of John Wiley and Sons.*

Table 4. Definitions of normal liver fat using liver histology, ^{1}H-MRS, MRI, CT and US

	Subjects	Definition of normal liver fat
Histology		
Kleiner DE et al. 2005	576 adults and 162 children	Macroscopic fat in <5% of hepatocytes
Brunt EM et al. 2011	976 adults	Macroscopic fat in <5% of hepatocytes
Bedossa P et al. 2012	679 morbidly obese subjects	Macroscopic fat in <5% of hepatocytes
^{1}H-MRS		
Szczepaniak LS et al. 2005	345 healthy subjects, population-based study	<5.56%
Petersen KF et al. 2006	170 healthy subjects	<3.0%
MRI		
Fishbein MH et al. 1997	28 healthy subjects	<9.0%
CT		
Piekarski J et al. 1980	100 healthy subjects	50-57 HU or 8-10 HU higher than spleen
US		
Joseph AE et al. 1978	60 adults referred to gastroenterologists	Absence of echogenicity or brightness of the liver
Saverymuttu SH et al. 1986	490 adults referred to gastroenterologists	Absence of echogenicity or brightness of the liver

^{1}H-MRS, proton magnetic resonance spectroscopy; CT, computed tomography; MRI, magnetic resonance spectroscopy; US, ultrasound. Adapted from Petäjä EM & Yki-Järvinen H. Int J Mol Sci 2016; 17: 633 and reproduced with the permission of MDPI (Creative Commons Attribution Licence).

5.3. DEFINITIONS OF NORMAL LIVER FAT (II)

The definitions of normal liver fat that were found in the systematic review are shown in Table 4. The biochemical definition of normal liver fat determined in 107 cadavers was 5.5 g of triglycerides in 100 g of wet liver tissue (Donhoffer, 1974)

5.3.1. Histology of liver biopsy

Histologically, normal liver fat is defined as less than 5% of hepatocytes containing macrovesicular steatosis (Kleiner et al. 2005; Bedossa *et al.*, 2012; Brunt and Tiniakos, 2010) (Table 4). Steatosis was graded by the pathologist from 0 to 3 based on its severity: grade 0 (normal): <5%; grade 1 (mild): 5% to 33%; grade 2 (moderate): 34% to 66%; and grade 3 (severe): ≥67% of hepatocytes characterised by macroscopic steatosis (Kleiner et al. 2005). Kleiner et al. developed the NAS score in 576 adults and 162 children of whom liver biopsy was obtained (Kleiner et al. 2005), and 58 of the adults and 5 of the children had <5% macroscopic liver fat. Bedossa et al. developed the SAF score in 679 morbidly obese subjects undergoing bariatric surgery, and of them 158 subjects had <5% of macroscopic liver fat (Bedossa *et al.*, 2012). No population-based studies exist, as obtaining a liver biopsy without a clinical indication would be unethical.

5.3.2. ^{1}H-MRS

Using ^{1}H-MRS, normal liver fat content has only been studied in one population-based study, the DHS. Normal liver fat was defined as the upper 95th percentile of liver fat content in healthy subjects. ^{1}H-MRS was performed on 2349 subjects, of whom 345 were considered healthy based on the following criteria: no history of liver disease or risk factors for hepatic

steatosis (alcohol consumption ≤30 g/day in men, ≤20 g/day in women, BMI <25 kg/m², normal fasting serum glucose, non-diabetic and normal serum ALT [≤30 U/l in men, ≤19 U/l in women]). The upper 95th percentile of liver fat content in healthy subjects was 5.56% (Szczepaniak *et al.*, 2005) (Table 4). This criterion is recognised as the standard criteria for the diagnosis of NAFLD using ^{1}H-MRS. Another study comprised 170 Caucasian subjects described as young, lean and healthy, but the criteria were not presented (Petersen *et al.*, 2006). The upper 95th percentile of liver fat content using ^{1}H-MRS was 3.0% (Table 4).

^{1}H-MRS-determined liver fat corresponds well to triglyceride content measured in a liver biopsy (r=0.90, p<0.001) (Thomsen *et al.*, 1994). The relationship between liver fat content evaluated by ^{1}H-MRS and percentage of macroscopic liver fat was analysed in three studies, which included 13 (Kotronen, Vehkavaara, *et al.*, 2007), 12 (Cowin *et al.*, 2008) and 50 (Noureddin *et al.* 2013) subjects. In the first two studies, the ^{1}H-MRS-determined normal liver fat in the DHS, i.e., 5.56%, corresponded to 15.7% (Kotronen, Vehkavaara, *et al.*, 2007) and 13.9% (Cowin *et al.*, 2008) of macroscopic steatosis. In the third study, histological grade 1 corresponded to 11% (7%–14%), grade 2 to 18% (14%–23%), and grade 3 to 25% (10%–28%) liver fat as measured by ^{1}H-MRS (Noureddin *et al.* 2013). Thus, normal liver fat content determined in liver histology, i.e. the percentage of hepatocytes with macroscopic steatosis, is approximately 2 to 3 times higher than the liver fat percentage measured by ^{1}H-MRS.

5.3.3. MRI

No population-based studies defining normal liver fat using the modified Dixon method or the MRI-PDFF method exist. The only study that has quantified liver fat in healthy subjects used the modified Dixon method, and defined normal liver fat as <9.0% in 28 healthy subjects (Fishbein *et al.*, 1997) (Table 4). No study assessing normal liver fat content in healthy subjects has been performed using MRI-PDFF, however the value of 5.6% derived in the DHS for ^{1}H-MRS (Reeder and Sirlin, 2010; Rehm *et al.*, 2015) or a value of 5.0% (Noureddin, *et al.* 2013; Yokoo *et al.*, 2009) are widely used.

Liver fat content measured using the MRI-PDFF method correlates closely with that determined from liver histology (8.9%–9.4% at grade 1, 15.8%–16.3% at grade 2, and 22.1%–25.0% at grade 3, p<0.0001) (Noureddin *et al.* 2013; Patel *et al.*, 2013; Permutt *et al.*, 2012; Tang *et al.*, 2013) and measured using ^{1}H-MRS (r=0.99) (Idilman *et al.*, 2015; Kang *et al.*, 2012; 2011; Noureddin *et al.* 2013). The 5% macroscopic liver fat corresponded to a PDFF value of 6% in 70 subjects (Idilman et al. 2013) and 6.4% in 12 adults and 65 children (Tang *et al.*, 2013). The best cut-off for MRI-PDFF-derived liver fat% to distinguish steatosis grade 0 (<5% steatosis) from grade 1–3 was defined in three studies: in 152 subjects as 5.2% of liver fat (Imajo *et al.*, 2016), in 97 subjects as 4.5% of liver fat (Kühn *et al.*, 2012) and in 56 subjects as 2.9% of liver fat (Kang *et al.*, 2012).

5.3.4. CT

In subjects with hepatic steatosis, the mean attenuation of the liver is lower than that of the spleen, and the liver appears darker than the spleen. In a study of 100 healthy adults, the attenuation in the healthy liver was 50 to 57 Houndsfield Units (HU) and 8 to 10 HU higher than the attenuation of spleen (Piekarski *et al.*, 1980) (Table 4). The attenuation value decreases by 1.6 HU for 1 mg of triglycerides per 1 g of liver tissue (Bydder *et al.*, 1981). Attenuation values <40 HU in the liver or 10 HU less in the liver than in the spleen are indicative of marked hepatic steatosis (>30%) (Hamer *et al.*, 2005).

5.3.5. US

In studies consisting of 60 (Joseph *et al.*, 1978) and 490 adults (Saverymuttu *et al.*, 1986) referred to gastroenterologist, normal liver fat was defined as absence of echogenicity or brightness of the liver (Table 4). Steatosis is scored semi-quantitatively as 'mild', 'moderate', or 'severe' based upon the visual assessment of hepatic echogenicity (Joseph *et al.*, 1978; Needleman *et al.*, 1986; Saverymuttu *et al.*, 1986). Mild steatosis is seen as a slight increase in liver echogenicity. In moderate steatosis, visualisation of intrahepatic vessels and the diaphragm is slightly impaired, and increased liver echogenicity was present. Severe steatosis is characterised as a marked increase in hepatic echogenicity, poor penetration of the posterior segment of the right lobe of the liver, and poor or no visualization of the hepatic vessels and diaphragm (Jain KA *et al.*, 1993). A meta-analysis of 44 studies comprising 4720 subjects concluded that US has a sensitivity of 85% and a specificity of 94% for detecting 20%–30% macroscopic steatosis (Hernaez *et al.*, 2011). The sensitivity and specificity were 65% and 81% for detecting 0%–5% steatosis and 93% and 88%, respectively, for detecting >10% steatosis.

5.4. INSULIN SENSITIVITY IN GENETIC NAFLD (II)

5.4.1. 'PNPLA3 NAFLD'

Table 5 summarises the 14 studies that include data on insulin sensitivity in carriers and non-carriers of the I148M variant (Del Ben *et al.*, 2014; Kantartzis *et al.*, 2009; Kotronen, Johansson, *et al.*, 2009; Lin *et al.*, 2011; Musso *et al.*, 2015; Park, Cho, Kwon, Prilutsky, Yun, Choi, Hwang, Lee, Kim, and Kong, 2015b; 2015a; Romeo *et al.*, 2008; Romeo, Sentinelli, Cambuli, *et al.*, 2010; Scorletti *et al.*, 2015; Valenti, Al-Serri, *et al.*, 2010; Valenti, Alisi, *et al.*, 2010; Verrijken *et al.*, 2013; Wagenknecht *et al.*, 2011; Wang *et al.*, 2011). These studies comprised 8425 subjects, and included obese and non-obese as well as diabetic and non-diabetic subjects. Three studies were performed in paediatric cohorts. In 12 studies, the carriers of the PNPLA3 I148M variant had significantly more liver fat or significantly higher prevalence of steatosis than non-carriers. HOMA-IR was used in 12 of the 14 studies as a marker of insulin sensitivity. Two studies used other methods: one OGTT (Kantartzis *et al.*, 2009) and the other fS-insulin concentration and the hyperinsulinemic clamp (Kotronen, Johansson, *et al.*, 2009). In 12 studies, no significant difference was found in HOMA-IR or other insulin sensitivity markers between carriers and non-carries. One study did not report the data but stated that the gene variant did not correlate with HOMA-IR or the insulin sensitivity index (Wagenknecht *et al.*, 2011). In one study, the homozygous carriers had significantly lower HOMA-IR than the non-carriers or the heterozygous carriers despite higher prevalence of steatosis (Park JH *et al.*, 2015). Serum triglycerides were reported in 12 studies. In 9 of these, there was no difference in serum triglyceride concentrations. Two studies found the carriers of the variant to have lower triglyceride concentrations and one study found the carriers to have higher triglyceride concentrations than the non-carriers.

5.4.2. 'TM6SF2 NAFLD'

Table 6 summarizes seven studies that have reported data on liver fat content and insulin sensitivity in carriers and non-carriers of the TM6SF2 E167K allele (Eslam *et al.*, 2016; Goffredo *et al.*, 2016; Grandone *et al.*, 2016; Kozlitina *et al.*, 2014; Scorletti *et al.*, 2015; Sookoian *et al.*, 2015; Zhou *et al.*, 2015). The studies comprised 7845 subjects and included two

Table 5. Insulin sensitivity in studies comparing liver fat between PNPLA3 I148M variant carriers (IM, MM) and non-carriers (II)

Cohort	N	BMI (kg/m^2)			Liver fat			Insulin sensitivity (HOMA-IR)			S-Triglycerides (mmol/l)		
		II	IM	MM	II	IM	MM	II	IM	MM	II	IM	MM
Multiethnic[1] (Caucasian, African and Hispanic American)	2111	30.4 31.6 29.6	31.1 32.0 28.8	30.0 32.2 28.8	3.7 [a] 3.1 3.5	4.6 [a] 4.8 3.7	7.7*** [a] 4.8*** 3.5***	3.3 3.3 2.3	3.5 3.3 2.4	3.3 4.4 2.0	1.32 0.97 1.25	.35 0.97 1.21	1.41 1.02 0.90
German [2]	330	29.9	29.1	28.7	5.4	6.0 [a]	7.2*** [a]	12.6[v,z]	12.9[v,z]	12.9[v,z]	NA	NA	NA
Finnish [3]	291	30.5	29.1	28.7	9.0 [a]	10.4* [a]	14.1** [a]	72[y,z]	72[y,z]	74[y,z]	1.82	1.60	1.52
British [4]	98	34.6	33.2	31.7	26.7 [a]	28.8 [a]	33.5 [a]	2.4	3.1	2.6	1.60	1.70	1.40
Multiethnic [5] (Hispanic and African American)	1214	NA[x]	NA[x]	NA[x]	57 [b] 55	55 [b] 51	46*** [b] 47***	NA[x]	NA[x]	NA[x]	NA[x]	NA[x]	NA[x]
Dutch [6]	470	37.7	37.6	37.6	66% [c]	78% [c]	100%*** [c]	2.7	2.8	2.9	1.42	1.47	1.46
Italian [7]	61	25.7	25.9		16% [d]	32%* [d]		3.4	4.7		1.13	1.15	
Italian [8]	253	30.7	30.7	29.8	44% [c]	48% [c]	63%** [c]	3.9	4.0	5.2	1.64	1.85	1.79
Italian [9]	211	32.1	30.4	31.7	4 [e]	4 [e]	4 [e]	3.5	3.5	2.8	1.77	1.59	1.26 **
Taiwanese [10]	879	23.3	23.6	23.6	13% [f]	19% [f]	23%* [f]	1.4	1.5	1.5	1.11	1.16	1.38 *
South Korean [11]	1363	24.7	24.4	23.9**	38% [f]	45% [f]	54%* [f]	2.3	2.1	1.6**	1.54	1.38	1.31 **
Taiwanese, pediatric [12]	520	26.3	26.2	25.9	21% [f]	13% [f]	30%** [f]	2.4	2.5	1.7	1.11	1.03	0.94
Italian, pediatric [13]	475	NA	NA	NA	13% [f]	19% [f]	41%* [f]	3.3	3.0	3.0	0.56	0.56	0.53
Italian, pediatric [14]	149	95.2°	95.0°	94.1°	70% [h] 30% 0%	7% [h] 78% 15%	4%*** [h] 4% 92%	2.5	2.7	2.4	1.28	1.19	1.39

*Data are presented as mean or median. *p<0.05, **p<0.01, ***p<0.0001 in ANOVA or unpaired t-test. [1]H-MRS, proton magnetic resonance spectroscopy; BMI, body mass index; CT, computed tomography; HOMA-IR, homeostasis model assessment of insulin resistance; MRI, magnetic resonance spectroscopy NA, not available; OGTT, oral glucose tolerance test; PNPLA3, patatin-like phospholipase domain containing 3; US, ultrasound. ° BMI centiles. [a] [1]H-MRS (liver fat%), [b] CT (liver attenuation, HU), [c] Histology (prevalence of steatosis, %),[d] Histology (% hepatocytes steatotic), [e] US (severity of steatosis by Hamaguchi score, 3–4= moderate), [f] US (prevalence of steatosis, %), [g] MRI (prevalence of steatosis, %), [h] Histology (severity of steatosis, grade 1/2/3). [v] OGTT (arbitrary unit), [y] fasting serum insulin (pmol/l), [z] hyperinsulinemic clamp also performed, data not in the table. [x] Data not shown, they reported that genetic variation in PNPLA3 at rs738409 did not correlate with HOMA-IR, insulin sensitivity index, BMI or S-triglycerides.[1]*Romeo *et al.*, 2008, [2] Kantartzis *et al.*, 2009, [3] Kotronen, Johansson, *et al.*, 2009, [4] Scorletti *et al.*, 2015, [5] Wagenknecht *et al.*, 2011, [6] Verrijken *et al.*, 2013, [7]Musso *et al.*, 2015, [8] Valenti, Al-Serri, *et al.*, 2010, [9] Del Ben *et al.*, 2014, [10] Wang *et al.*, 2011, [11]Park, *et al.*,2015 [12] Lin *et al.*, 2011, [13] Romeo, Sentinelli, Cambuli, *et al.*, 2010, [14] Valenti, Alisi, *et al.*, 2010. Adapted from Petäjä EM & Yki-Järvinen H. Int J Mol Sci 2016; 17: 633 and reproduced with the permission of MDPI (Creative Commons Attribution Licence)..*

Table 5. Insulin sensitivity in studies comparing liver fat between PNPLA3 I148M variant carriers (IM, MM) and non-carriers (II)

Cohort	N	BMI(kg/m2)		Liver fat		Insulin sensitivity HOMA-IR		S-Triglycerides (mmol/l/l)	
		EE	EK+KK	EE	EK+KK	EE	EK+KK	EE	EK+KK
Multiethnic [1] (Caucasian, African and Hispanic Americans)	4587	29.6	28.5/31.8	3.5 [a]	4.4/15.7 *** [a]	3.0	2.9/4.6	1.39	1.33/1.47*
Finnish [2]	300	33.7	32.5	6.8 [a]	11.2 * [a]	3.0	2.9	1.40	1.50
British [3]	98	32.6	35.4	28.5 [a]	29.0 [a]	2.7	4.0	1.60	1.50*
Argentineans [4]	361	29.8	30.2	NA	NA	3.1	3.0	1.87	1.31
Multiethnic [5] (Caucasian, Asian, Hispanic)	502	32.2	31.2/30.8	S0:3% [b] S1:50% S2:27% S3:20%	S0:0%/0% [b] S1:35%/45% S2:40%/20% S3:25%/35%	3.4	2.8/2.8	1.70	1.36/1.08 **
Multiethnic, pediatric [6] (Caucasian, African and Hispanic Americans)	957	33.0	32.6	6.7 [c ^]	11.1 ** [c ^] *	1.9 [x]	2.0 [x]	1.20	1.21
Italian, pediatric [7]	1010	2.9°	2.9°	47% [d]	89% ** [d]	5.6	4.6	1.12	1.02

*Data are presented as mean or median. *p<0.05, **p<0.01, ***p<0.0001 in ANOVA or unpaired t-test. [1]H-MRS, proton magnetic resonance spectroscopy; BMI, body mass index; CT, computed tomography; HOMA-IR, homeostasis model assessment of insulin resistance; MRI, magnetic resonance spectroscopy NA, not available; OGTT, oral glucose tolerance test; TM6SF2, transmembrane 6 superfamily member 2; US, ultrasound*

^ Liver fat content available on 454 subjects, BMI, insulin sensitivity and S-Triglycerides on 957 subjects. °BMI-SDS [a] [1]H-MRS (liver fat content%), [b] Histology, prevalence of each steatosis grade, [c] US, [d] MRI-PDFF (liver fat%, n=454); [x] OGTT (whole-body insulin sensitivity index). [1] Kozlitina *et al.*, 2014, [2] Zhou *et al.*, 2015, [3] Scorletti *et al.*, 2015 [4] Sookoian *et al.*, 2015, [5]Eslam *et al.*, 2016, [6] Goffredo *et al.*, 2016, [7]Grandone *et al.*, 2016 *Adapted from Petäjä EM & Yki-Järvinen H. Int J Mol Sci 2016; 17: 633 and reproduced with the permission of MDPI (Creative Commons Attribution Licence).*

paediatric cohorts. In six of these studies, the carriers had a significantly higher liver fat content, determined by ^{1}H-MRS or MRI, or higher prevalence of steatosis, determined by histology or US, than non-carriers. One study found no difference in liver fat content between the two groups (Scorletti *et al.*, 2015). There was no difference in insulin sensitivity, determined by HOMA-IR or OGTT, between the carriers and non-carriers. Serum triglyceride concentrations were lower in three studies (Eslam *et al.*, 2016; Grandone *et al.*, 2016; Scorletti *et al.*, 2015), similar in three studies (Goffredo *et al.*, 2016; Kozlitina *et al.*, 2014; Zhou *et al.*, 2015) and higher in one study (Sookoian *et al.*, 2015) in the TM6SF2 E167K allele carriers compared to non-carriers.

5.5. REFERENCE VALUES FOR HOMA-IR (III)

Characteristics of the healthy subjects in the two population-based cohorts (n=1167 for the FINRISK/DILGOM and n=459 for the FIN-D2D cohort) are shown in Table 2.

5.5.1. Reference value for HOMA-IR in two population-based cohorts

The upper reference limit (95th percentile [90% CI)]) of HOMA-IR was 1.9 (1.8 –2.0) in the FINRISK-/DILGOM cohort and 2.0 (1.9–2.2) in the FIN-D2D cohort. There was no significant difference between the HOMA-IRs in either cohort between genders (Table 7). In both cohorts, men were slightly more obese and older (III, Table 1) and to correct for this, age- and BMI-adjusted HOMA-IRs were calculated. The adjusted HOMA-IRs were very similar compared to the unadjusted values (in the FINRISK/DILGOM 1.0 [0.9–1.1] in all subjects, 1.0 [0.9–1.1] in women and 1.08 [1.0–1.1] in men, p=0.0007 between genders, and in the FIN-D2D 1.1 [1.0–1.2] in all subjects, 1.1 [1.0–1.2] in women and 1.1 [1.1–1.2] in men, p=0.0019). No significant difference was observed in the clinical characteristics between the carriers and non-carriers of the PNPLA3 I148M variant (III, Supp. Table 1). The 95th percentiles of HOMA-IR between the PNPLA3 I148M non-carriers and carriers were similar (Table 7).

5.6. OPTIMAL HOMA-IR CUT-OFF FOR NAFLD (III)

Characteristics of the non-diabetic subjects (n=368) are shown in Table 2.

5.6.1. HOMA-IR corresponding to normal liver fat

HOMA-IR and liver fat content correlated significantly (r=0.67, p<0.0001 in all subjects; r=0.67, p<0.0001 in men r=0.66, p<0.0001 in women). In linear regression analysis, the HOMA-IR corresponding to NAFLD as defined as in the DHS (liver fat=5.56%) was 2.0 (1.9–2.1) (III, Fig. 2a) in all subjects, 1.9 (1.8–2.1) in women and 2.1 (1.9–2.2) in men (slopes p=0.79, elevations p=0.75).

The HOMA-IR corresponding to normal liver fat content was significantly higher (2.1 [2.0–2.2] vs. 1.8 [1.6–1.9], slopes p=0.99, intercepts p=0.007) in non-carriers than carriers of the PNPLA3 I148M variant (III, Fig. 2b) (correlation r=0.68, p<0.0001 in non-carriers; r=0.66, p<0.0001 in carriers of the variant). This causes the variant allele carriers to be significantly more insulin sensitive than the non-carriers for a given liver fat content.

The upper 95th percentile for liver fat in the 96 healthy (defined as in the DHS) (n=96) subjects was 5.9%.

5.6.2. Optimal HOMA-IR cut-off to diagnose NAFLD

ROC analysis was performed to define the optimal cut-off for HOMA-IR to distinguish NAFLD from non-NAFLD. Subjects were randomised into discovery (2/3) and validation (1/3 of subjects) groups that matched with respect to clinical characteristics. The AUROC for HOMA-IR was 0.88 (95% CI 0.84–0.92) in the discovery group (Fig. 2). The optimal cut-off for NAFLD based on the Youden Index was a HOMA-IR of 1.9. This cut-off had a sensitivity of 87%, specificity of 79%, negative predictive value (NPV) of 92% and positive predictive value (PPV) of67%. The results were similar for the validation group (Fig. 2): AUROC 0.80 (0.70–0.88), sensitivity 68%, specificity 82%, NPV 81%, and PPV 70%, and for all subjects: AUROC 0.85 (0.80–0.89), sensitivity 80%, specificity 80%, NPV 88%, and PPV 68%. The AUROC for bootstrap samples was 0.88 (0.82–0.92) and the overall estimate of optimism was 0.00079. Neither gender (p=0.50) nor *PNPLA3* genotype (p=0.21) significantly improved the AUROC.

5.6.3. Inter-laboratory variation in insulin assays and HOMA-IR

Table 2 shows the clinical characteristics of 10 subjects. The mean insulin concentrations from seven laboratories ranged from 3.0 to 15.2 mU/l, glucose from 4.7 to 6.1 mmol/l and HOMA-IR from 0.69 to 4.0 (Table 8). Freezing and thawing the serum the same day had no impact on fasting insulin (8.8±4.8 mU/l vs. 9.0±4.9 mU/l, p=0.077). Serum insulin concentrations decreased over time when stored at -80°C degrees for two weeks (7.6±4.3 mU/l vs. 9.0±4.9 mU/l, p=0.005).

Figure 2.

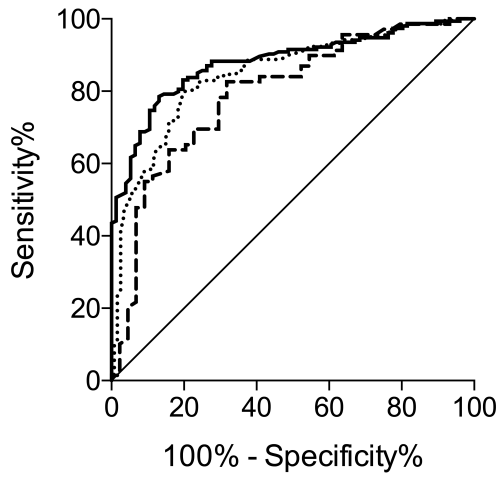

Figure 2. AUROC for HOMA-IR and NAFLD. The AUROC for HOMA-IR was 0.88 (95% CI 0.84-0.92) in the discovery group (solid line), 0.80 (0.70-0.88) in the validation group (dashed line) and 0.85 (0.80-0.89) in all individuals (dotted line). Reproduced from Isokuortti E *et al*., *Diabetologia* 2017:60;1873-1882 and with the permission of Springer Berlin Heidelberg.

Table 8 presents results from simultaneous analysis of samples stored at -80°C for two weeks in seven participating laboratories. The CV of fasting insulin and glucose measured in the seven laboratories averaged 25.4% and 4.6%, respectively. The CV of HOMA-IR was 25.0% (Table 8). All the CVs with the exception of ferritin were significantly (p<0.01) lower than the CV for fasting insulin.

Correlation coefficients between HOMA-IR measured in Helsinki and in other laboratories are shown in Table 9. The slopes differed between Helsinki and Newcastle, Mainz and Pisa clinical lab, and the intercepts with Torino. The HOMA-IR value of 2.0 measured in Helsinki corresponded to HOMA-IRs of 1.3, 1.6, 1.8, 1.8, 2.0 and 2.1 at the six other laboratories.

Table 7. The upper 95th percentile (90% CI) of HOMA-IR in healthy subjects of the FINRISK/DILGOM and FIN-D2D cohorts.

	FINRISK/DILGOM (n=1167)	FIN-D2D (n=459)
All subjects	1.9 (1.8-2.0)	2.0 (1.9-2.2)
Women	1.8 (1.8-2.0)	2.0 (2.0-2.4)
Men	1.9 (1.8-2.0)	2.0 (1.9-2.3)
PNPLA3^{I148II}	1.9 (1.8-2.0)	2.0 (2.0-2.2)
PNPLA3$^{I148IM/MM}$	1.8 (1.7-2.0)	2.0 (1.9-2.5)

CI, confidence interval; PNPLA3, patatin-like phospholipase domain containing 3 gene

Table 8. Inter-laboratory variation in 7 European laboratories

	Range	Mean	SD	CV (%)
fS-Insulin (mU/l)	3.0-15.2	6.79	1.59	25.4
fP-Glucose (mmol/l)	4.7-6.1	5.5	0.25	4.6
HOMA-IR	0.69-3.96	1.7	0.4	25.0
fP-Total cholesterol (mmol/l)	3.61-6.55	4.81	0.35	7.4
fP-HDL cholesterol (mmol/l)	0.91-2.35	1.74	0.13	7.0
fP-LDL cholesterol (mmol/l)	0.89-4.10	2.46	0.30	12.8
fP-Triglycerides (mmol/l)	0.59-2.79	1.36	0.12	8.3
fP-Albumin* (g/l)	37-47	42	3.2	7.7
fP-Ferritin* (μg/l)	14-140	62	10.7	19.1
fP-ALT (U/l)	13-41	23	2.5	11.6
fP-AST (U/l)	19-71	31	3.7	11.7
fP-GGT (U/l)	10-52	20	2.5	11.3

**not available from Pisa Research laboratory. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CV, coefficient of variation; fP, fasting plasma; fS, fasting serum; GGT, gamma-glutamyltransferase; HbA_{1c}, glycated haemoglobin A_{1c}; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; SD, standard deviation*

Table 9. Comparison of HOMA-IR measured in Helsinki and 6 other laboratories.

	Pearson r	*P* value	Slopes *P* value	Intercepts *P* value	HOMA-IR 2.0 in Helsinki corresponds to
Newcastle, UK	0.93	<0.0001	<0.0001	ns	1.3
Mainz, Germany	0.96	<0.0001	0.0004	ns	1.8
Paris, France	0.99	<0.0001	ns	ns	2.0
Pisa (Clinical), Italy	0.96	<0.0001	0.009	ns	1.8
Pisa (Research), Italy	0.87	0.001	ns	ns	2.1
Torino, Italy	0.97	<0.0001	ns	0.0005	1.6

5.7. fS-pIGFBP-1 IN NAFLD (IV)

Clinical characteristic of 378 subjects are shown in Table 2. The discovery (n=252) and validation (n=126) groups were comparable with respect to their clinical and biochemical parameters as well as *PNPLA3* genotype at rs739409 (IV, Table 1). 46% of the subjects had NAFLD. The median fS-pIGFBP-1 concentration was 58 (32–106) µg/l.

5.7.1. Correlation of liver fat and associated factors

The variables were divided into groups measuring the same biological phenomena, as body composition, liver enzymes, lipids, glycaemia and insulinemia. In the discovery group, liver fat content was significantly inversely correlated with fS-pIGFBP-1 concentration (ρ=-0.21, p=0.0009) and significantly positively correlated with age (ρ=0.25, p<0.0001), male gender (ρ=0.14, p=0.02) and the PNPLA3 I148M allele (ρ=0.16, p=0.01). Significant correlations were also found between liver fat content and liver enzymes (S-ALT [ρ=0.46, p<0.0001] and S-AST [ρ=0.37, p<0.0001]), measures of glycaemia (fP-glucose [ρ=0.42, p<0.0001], and HbA_{1c} [ρ=0.40, p<0.0001]) and measures of insulinemia (fS-insulin [ρ=0.46, p<0.0001] and fP-C-peptide [ρ=0.32, p<0.0001]). Of lipids, fS-triglycerides correlated positively (ρ=0.40, p<0.0001) and fS-HDL cholesterol inversely (ρ=0.32, p<0.0001) with liver fat content. Measures of body composition also correlated positively with liver fat content (waist-to-hip ratio [ρ=0.41, p<0.0001], body weight [ρ=0.20, p=0.002], BMI [ρ=0.17, p=0.005], waist circumference [ρ=0.28, p<0.0001], and body fat percentage [ρ=0.17, p=0.022], The variables with the best predictive value within each group of biological phenomena in the discovery group were thus S-ALT, fP-glucose, fS-insulin, fS-triglycerides and waist-to-hip ratio. The correlation coefficient between fS-insulin and fS-pIGFBP-1 in all subjects was ρ=-0.51, p<0.0001.

5.7.2. Prediction of liver fat content

The above-mentioned variables with the best correlations to liver fat content, among them age, gender, fS-pIGFBP-1 and *PNPLA3* genotype at rs738409, were evaluated by multivariate linear regression analysis to find independent associations in the discovery group. The significant variables and their possible interactions were examined. The final variables for multiple linear regression analysis were derived using a backward stepwise regression method. The significant variables were age, fS-pIGFBP-1, an interaction term (age x fS-pIGFBP-1), fS-ALT, waist-to-hip ratio, fP-glucose and fS-insulin, forming the '% Liver fat equation' (adjusted R^2=0.44, p<0.0001). The adjusted R^2 was 0.49 in the validation group and 0.47 in all subjects. If fS-pIGFBP-1 was excluded, adjusted R^2 was 0.46 in all subjects (p<0.05 vs. the best model), and if fS-insulin was excluded, the adjusted R^2 was 0.44 in all subjects (p<0.0001 vs. the best model). The correlation coefficient between predicted liver fat content using the '% Liver Fat Equation' (IV, Table 3) and liver fat measured using ^{1}H-MRS was ρ=0.62, p<0.0001 (Fig. 3).

The '% Liver Fat Equation' predicted liver fat significantly better than liver enzymes: AST only (adjusted R^2=0.15), ALT only (adjusted R^2=0.25), or both (adjusted R^2=0.25, p<0.0001 for all comparisons). The AUROC (95% CI) to distinguish NAFLD from non-NAFLD with the '% Liver Fat Equation' was 0.84 (0.80–0.88). This was significantly better than that of the FLI (0.72 [0.67–0.77], p<0.0001) or the HSI (0.62 [0.57–0.68], p<0.0001).

The best predictors in univariate analysis within each group along with age, gender, fS-pIGFBP-1 and *PNPLA3* genotype at rs738409 were also subjected to Random Forest modelling for prediction of liver fat (IV, Fig. 2). S-ALT, waist-to-hip ratio, fS-insulin, fS-triglycerides and fS-pIGFBP-1 were identified as the best five variables to explain variation in liver fat content (adjusted R^2=0.39 in all subjects).

Figure 3.

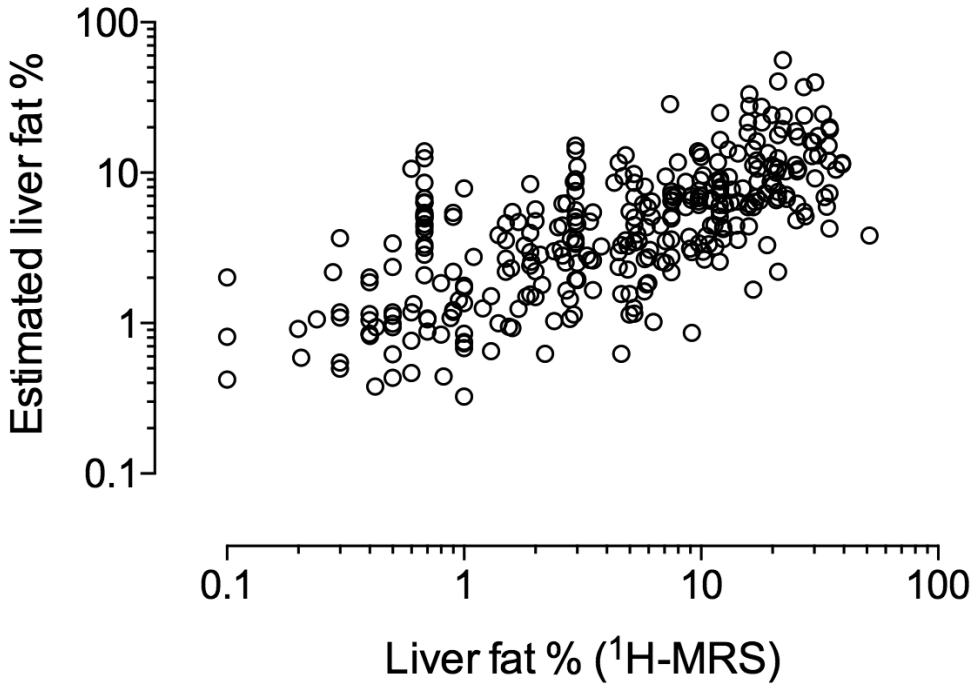

Figure 3. Correlation between liver fat percentages measured by ^{1}H-MRS and estimated by '% Liver Fat Equation' (ρ=0.62, p<0.0001). Adapted from Petäjä EM *et al., Sci Rep* 2016:6;24740 and reproduced with the permission of Nature Research Group.

6 DISCUSSION

The prevalence of both MetS and NAFLD increases with obesity, and once fatty, the insulin-resistant liver overproduces the two key components of MetS, glucose and triglycerides (Yki-Järvinen, 2014). Almost half of people with NAFLD carry at least one variant (G) allele at rs738409 in the *PNPLA3* gene, which is associated with high liver fat content, but is not accompanied by features of MetS (Sookoian and Pirola, 2011). All forms of NAFLD increase the risk of NASH, cirrhosis, and HCC. The present studies were undertaken to examine aspects of pathogenesis and diagnosis of NAFLD.

6.1. ADIPOCYTE SIZE IN NAFLD

In study I examining whether SC adipocyte size is a determinant of liver fat, the latter correlated with measures of obesity and insulin resistance, dyslipidemia, liver enzymes, *PNPLA3* genotype and SC adipocyte size. Even though such univariate associations have been reported previously (Koska *et al.*, 2008; Kotronen, Westerbacka, *et al.*, 2007; Romeo *et al.*, 2008), the present data are novel as the independent contributions of the impact of SC adipocyte size as well as that of the *PNPLA3* genotype on liver fat were examined. Prior to this study, two human studies have reported a positive correlation between SC adipocyte size and liver fat. O'Connell *et al.* reported a positive correlation between adipocyte size with the degree of hepatic steatosis as determined by histological assessment of samples from 19 subjects undergoing bariatric surgery (O'Connell *et al.*, 2010). Koska *et al.* measured, in 53 obese Pima Indians, SC adipocyte size and the liver/spleen attenuation ratio using CT, which provided a qualitative rather than quantitative measure of liver fat (Koska *et al.*, 2008). Neither study determined the independence of the association or considered the *PNPLA3* genotype. Since article I was published, two more studies have assessed liver steatosis and adipocyte size. Wree *et al.* studied 94 morbidly obese subjects undergoing bariatric surgery and found visceral adipocyte diameter to correlate positively with increasing NAS score (Wree *et al.*, 2014). They did not report on whether the association was independent of other factors influencing liver fat content nor the relationship between adipocyte size and steatosis score (Wree *et al.*, 2014). Jansen *et al.* measured liver fat content using ^{1}H-MRS in 27 obese subjects with type 2 diabetes (mean BMI 31 kg/m^2) (Jansen *et al.*, 2013). They found that liver fat content did not correlate with SC adipocyte size or measures of obesity (BMI, SC adipose tissue volume) but did correlate with markers of adipose tissue inflammation (Jansen *et al.*, 2013). The lack of positive association between liver fat content and SC adipocyte size may be due to the difference in study populations (non-diabetic vs. diabetic) and the small sample size.

To study independent associations in multivariate analyses, results of the univariate analyses and pre-existing knowledge of causes and consequences of NAFLD were examined. The *PNPLA3* genotype (Romeo *et al.*, 2008), age and gender (Kotronen, Westerbacka, *et al.*, 2007) and BMI (or fat mass or abdominal SC fat mass) were considered to be causes of variation in liver fat, while mild fasting hyperglycaemia and hypertriglyceridemia (resulting in low HDL cholesterol) were consequences rather than causes of hepatic insulin resistance. As discussed in the review of the literature (section 2.1.3.1), regarding the impact of gender on liver fat, women have significantly more SC and less IA fat for a given liver fat content than men do (Kotronen, Westerbacka, *et al.*, 2007). However, men and women have equal amounts of liver fat if adjusted for the amount of IA adipose tissue volume. In the current study, gender did not remain as

an independent predictor in the face of the other correlates of liver fat content. In multiple linear regression analyses, addition of adipocyte size into the model consisting of age, BMI, IA/SC adipose tissue ratio and the *PNPLA3* genotype increased the explanatory power of the model from 42% to 53%, suggesting that adipocyte size contributes in a highly significant manner to the inter-individual variation in liver fat, although a cross-sectional analysis cannot prove cause and effect.

Adipocyte hypertrophy rather than obesity itself may be a key factor in the pathogenesis of adipose tissue inflammation. Adipocytes pushed to the limits of their ability to store lipids reach a genetically determined 'critical size', which leads to hypoxic stress and the release of higher levels of proinflammatory mediators, such as leptin and resistin, and lower amounts of adiponectin (Kershaw and Flier, 2004; Skurk *et al.*, 2007) and the ensuing adipose tissue insulin resistance. Hypertrophied adipocytes secrete a surplus of pro-inflammatory cytokines, such as TNF-α and MCP-1 (Guilherme *et al.*, 2008), that function as chemoattractants, leading to accumulation of macrophages and inflammation in adipose tissue (Zeyda and Stulnig, 2009). In line with this, adipocyte size is closely correlated with the number of macrophages in adipose tissue (Weisberg *et al.*, 2003). Mice that lack HSL have hypertrophied adipocytes and macrophage infiltration in their adipose tissue despite being non-obese (Cinti *et al.*, 2005), while apoptosis of hypertrophied adipocytes also promotes accumulation of macrophages in adipose tissue (Alkhouri *et al.*, 2010). Inflammatory cytokines, such as TNF-α, disrupt insulin action in adipocytes, resulting in increased lipolysis and decreased triglyceride storage (Rydén and Arner, 2007). Hypertrophied adipocytes have higher lipolytic capacity than smaller adipocytes (Foley *et al.*, 1997; Laurencikiene *et al.*, 2011). *In vitro*, insulin sensitivity of human adipocytes was already 50 years ago shown to be inversely proportional to adipocyte size (Salans *et al.*, 1968). As discussed earlier, insulin resistance in adipose tissue increases circulating FFA (Salans *et al.*, 1968), the key main substrate of intrahepatocellular triglycerides both in the fasting and post-prandial states (Donnelly *et al.*, 2005).

The liver is a physiologically likely mechanistic link between adipocyte size and alterations in circulating markers of the MetS, since fatty liver overproduces them (Kotronen and Yki-Järvinen, 2008; Kotronen *et al.*, 2011). Thus, the independent relationship between adipocyte size and liver fat helps to explain why adipocyte hypertrophy correlates with features of insulin resistance independent of obesity and body composition (Hoffstedt *et al.*, 2010; Weyer *et al.*, 2000). The association between adipocyte size and liver fat may also help explain why both adipocyte hypertrophy (Lönn *et al.*, 2010; Weyer *et al.*, 2000) and liver fat (Lallukka and Yki-Järvinen, 2016) predict development of type 2 diabetes independent of obesity, gender and age.

The current study is the first to study the relationship between SC adipocyte size and liver fat content independent of other known causes of liver steatosis. This is also the largest study reporting a positive correlation between adipocyte size and liver fat, as the previous studies comprised 53 (Koska *et al.*, 2008) and 19 (O'Connell *et al.,* 2010) subjects. This study is cross-sectional and hence cannot prove cause and consequence. Further studies are needed to establish whether adipocyte hypertrophy is in fact a predictor of development of NAFLD. Regarding the method used to determine adipocyte size, we measured the mean diameter of 100 SC adipocytes. This method is used frequently by others as well (Arner *et al.*, 2010; Björnheden *et al.*, 2004; Lönn *et al.*, 2010;

Lundgren *et al.*, 2007) but provides a mean of less adipocytes than when using the osmium tetroxide and Counter method. Biopsies of intra-abdominal adipose tissue, although potentially interesting, were not taken from the healthy volunteers for ethical reasons, and hence we were not able to compare whether the association between IA adipocyte size and liver fat content differs from that of SC adipocyte size.

6.2. DEFINITIONS OF NORMAL LIVER FAT

As discovered in study II, the definitions of normal liver fat content vary, and are dependent on the method used, as they measure steatosis differently. In histologic assessment, a pathologist visually evaluates the percentage of hepatocytes with macroscopic lipid droplets, whereas ^{1}H-MRS quantifies the hepatic triglyceride content in a single voxel. MRI-PDFF also quantifies the hepatic triglyceride content, but does so on of all of the lobes of the liver. CT evaluates steatosis as the attenuation of liver and compared to that of the spleen, and US as a diffuse increase in parenchymal brightness and echogenicity of liver, thus give a qualitative estimate of degree of steatosis.

^{1}H-MRS remains the gold standard for quantifying liver fat content and is the most accurate method for assessing steatosis (Bohte *et al.*, 2011). In the population-based DHS, the upper 95th percentile of the liver fat percentage in healthy subjects was 5.56% (Szczepaniak *et al.*, 2005). In the present study, using the same criteria for the definition of healthy, and conducted on a selected, rather than population-based cohort, we found the 95th percentile to be 5.9%. In our Liver Fat Cohort, the prevalence of NAFLD was 35%, which is equivalent to the population prevalence of NAFLD at 33% in the DHS (Browning *et al.*, 2004). Another study that included 170 healthy subjects found the 95th percentile to be 3.0% (Petersen *et al.*, 2006). The authors speculated their lower value, compared to the DHS, might be because the subjects of their study might have been leaner, and the performance of ^{1}H-MRS scanning differed, with the liver fat content in the DHS possibly being overestimated (Petersen *et al.*, 2006). No population-based studies have been performed using the MRI-PDFF, and therefore no gold standard definition exists, even though the method is widely used for research purposes. Definitions based on the ^{1}H-MRS-derived 5.6% (Reeder and Sirlin, 2010; Rehm *et al.*, 2015) or 5.0% (Noureddin *et al.* 2013) are the most widely used.

Liver steatosis is subject to some intrahepatic variation (Bannas *et al.*, 2015). Bannas et al. studied 13 *ex vivo* human livers, of which multiple biopsies were obtained and on which ^{1}H-MRS and MRI-PDFF were performed. Depending on the location of the samples, histological macroscopic steatosis differed between 10% and 25%, liver fat content measured using MRI-PDFF from 10% to 23% and ^{1}H-MRS from 12% to 20%. However, intra-individually, the results of histological assessment remained mostly within the same steatosis grade (Bannas *et al.*, 2015).

None of the diagnostic cut-offs presented in Table 4 were based on metabolic or clinical outcomes of increased liver fat content. As the relationship between intrahepatic triglyceride content, measured using ^{1}H-MRS, and metabolic function in obese subjects is in fact linear (Kotronen, Westerbacka, *et al.*, 2007); Korenblat et al. 2008), it is uncertain which value of liver fat actually has prognostic significance.

Accurate quantification of steatosis is important for prospective longitudinal studies assessing the natural course of fatty liver, as well as treatment studies assessing the efficacy of medications (Chalasani *et al.*, 2012; Musso *et al.*, 2012; Noureddin *et al* 2013). For these purposes, US, and CT,

which assess steatosis qualitatively rather than quantitatively, are not applicable. US remains as a tool extensively used for screening purposes in the clinic, as more accurate methods are more costlier and not as widely available, even though its poor sensitivity in less than 30% of macroscopic liver fat is well known (Ryan *et al.*, 2002). Due to radiation exposure, using CT as a screening tool is not recommended, even though it is more widely available, faster to perform and cheaper than MRI-based methods (Fierbinteanu-Braticevici, 2010).

6.3. DEFINITIONS OF NORMAL INSULIN SENSITIVITY

6.3.1 Insulin sensitivity in 'Genetic NAFLD'

In the systematic review in study II, studies reporting markers of insulin sensitivity and liver fat 'PNPLA3 NAFLD' and 'TM6SF2 NAFLD' were identified. Regarding 'PNPLA3 NAFLD', 14 studies were identified. In 12 out of the 14, despite higher liver fat content or prevalence of hepatic steatosis, the carriers of PNPLA3 I148M variant had similar or better insulin sensitivity than non-carriers of the variant. As previously shown, 'PNPLA3 NAFLD', even though it increases the risk of NAFLD, is not associated with features of MetS (Kantartzis *et al.*, 2009; Kotronen, Johansson, *et al.*, 2009; Romeo *et al.*, 2008; Sookoian *et al.*, 2009). In study III, we found no difference in normal insulin sensitivity as defined by the 95th percentile of HOMA-IR in carriers and non-carriers of the PNPLA3 I148M variant in healthy subjects of two population-based cohorts. In non-diabetic subjects in whom liver fat content was measured using ^{1}H-MRS, normal liver fat content being 5.56% as in DHS (Szczepaniak *et al.*, 2005), the carriers of the variant had a significantly lower HOMA-IR in the face of similar liver fat content than the non-carriers. This is in line with the results from study II. Regarding the dissociation between hepatic steatosis and insulin resistance in 'PNPLA3 NAFLD', our laboratory recently discovered in 125 morbidly obese subjects undergoing bariatric surgery, that the livers of carriers of the PNPLA3 I148M variant are enriched with polyunsaturated triglycerides whereas other lipids remained unchanged (Luukkonen, Zhou, Sädevirta, *et al.*, 2016). In contrast, in NAFLD associated with insulin resistance, the liver was enriched with saturated and monounsaturated triglycerides and FFAs as well as dihydroceramides and ceramides, which are known to induce insulin resistance (Summers, 2006). This might in part explain why the 'Metabolic' but not 'PNPLA3 NAFLD' is associated with insulin resistance.

In the systematic review in study II, 7 studies that assessed both insulin sensitivity and liver fat in 'TM6SF2 NAFLD' were identified. In 5 out of 7 of the studies, the carriers of the *TM6SF2* E167K variant had higher liver fat content or higher prevalence of steatosis than the non-carriers without increased insulin resistance. In previous studies, the variant has been associated with lower circulating total and LDL cholesterol, and with lower triglyceride concentrations (Pirola and Sookoian, 2015) and with a lower risk of cardiovascular disease in carriers than non-carriers (Dongiovanni *et al.*, 2015; Pirola and Sookoian, 2015). This has been hypothesised as caused by an impairment in the lipidation of VLDL (Smagris *et al.*, 2016). No studies regarding insulin resistance in 'MBOAT7 NAFLD' so far exist, but this will certainly be of interest in the future.

Even though it has been shown that 'Genetic NAFLD' is not associated with insulin resistance, subjects with it are not completely protected against it, but instead may be susceptible to both disorders (Yki-Järvinen, 2014). Also, one may have more than one of the risk allele and be thus more susceptible to NAFLD. In 384 NAFLD patients and 384 age- and gender-matched

controls, when adding one risk allele of *PNPLA3* or *TM6SF2*, the odds ratio for the risk of NAFLD was 1.64 (95% CI 1.34–2.01), p<0.001. In the DHS, *PNPLA3, TM6SF2* and *MBOAT7* genotyping was performed on 2736 subjects, and liver fat content was shown to increase proportionally with increased number of risk alleles (Mancina *et al.*, 2016). They did not report whether this increased risk of hepatic steatosis was associated with increased risk of insulin resistance.

6.3.2 Reference value for HOMA-IR

In study III, the healthy subjects of two population-based cohorts were identified, and the upper reference limit (95th percentile [90% CI]) for HOMA-IR was 1.9 (1.8–2.0) in the FINRISK/DILGOM cohort and 2.0 (1.9–2.2) in the FIN-D2D cohort.

Three previous studies attempting to define a reference value HOMA-IR have been performed in healthy subjects. These studies included fewer subjects considered healthy (161 Japanese subjects, 161 Italian subjects and 312 Brazilian subjects) than in the present study (456 to 1167 subjects) (Bonora *et al.*, 1998; Geloneze *et al.*, 2006; Nakai *et al.*, 2002). In the Japanese study (Nakai *et al.*, 2002), the 90th percentile of HOMA-IR was 1.7, which is comparable to that found in the present study. The Italian study, however, included diabetic and hypertensive subjects who thus cannot be considered healthy. The 80th percentile of HOMA-IR was 2.77 (Bonora *et al.*, 1998). This study used the non-specific RIA by Linco Research Inc. (Missouri, US), which has given the highest insulin concentrations of several insulin assays tested (Manley *et al.*, 2007; 2008). Similarly this RIA was used in the Brazilian study (Geloneze *et al.*, 2006), and the 90th percentile of HOMA-IR was found to be equally high (2.71). Thus, the higher HOMA-IR values in these studies compared to the present study could be due to the inclusion of diabetic and hypertensive subjects in the Italian study and to both studies' due to use of a RIA that is no longer used in most laboratories (Manley *et al.*, 2007).

We found no significant differences in HOMA-IR percentiles between men and women in the healthy subjects in either population-based cohort (Table 7). The men were, however, slightly more obese and older than the women, which is why we also calculated age- and BMI-adjusted HOMA-IRs. After adjustment, the men had slightly higher HOMA-IRs than the women in both studies, but the differences in absolute units were trivial (0.02 in the FINRISK and 0.05 in the FIN-D2D study). Previous population-based studies including healthy subjects did not report HOMA-IR values separately for men and women (Bonora *et al.*, 1998; Geloneze *et al.*, 2006; Nakai *et al.*, 2002). Limitations of the use of HOMA-IR are discussed in section 6.4.2.

6.4 DIAGNOSIS OF NAFLD

6.4.1 HOMA-IR corresponding to normal liver fat content and the optimal HOMA-IR cut-off for NAFLD

Study III was undertaken to determine whether a single value of HOMA-IR could be used to clearly identify subjects with NAFLD and how HOMA-IRs between laboratories in different European countries compare to each other. In 368 subjects, whose liver fat content was determined using ^{1}H-MRS, a HOMA-IR of 1.9 was the best for discriminating subjects with NAFLD from those without based on the Youden Index. HOMA-IR of 2.0 corresponded to normal liver fat content of 5.56% defined as the upper limit of normal liver fat content in the DHS.

In keeping with the 95th percentile in the healthy subjects in the population-based cohorts, a HOMA-IR of 1.9 was the best for distinguishing subjects with and without NAFLD in ROC analysis (Fig. 2). This

value corresponds to the results in 88 healthy Brazilian subjects and 116 subjects with NAFLD diagnosed by ultrasound or biopsy (Salgado *et al.*, 2010). As determined by our study, the Brazilian study found a HOMA-IR of 2.0 (AUROC 0.84) to best distinguish between NAFLD and non-NAFLD subjects. Also, a study comprising 263 Columbian men found a HOMA-IR of 1.7 (AUROC 0.78) to be the cut-off for NAFLD (Perez *et al.*, 2011). No previous studies in Caucasian populations have been performed. In linear regression analysis, a HOMA-IR of 2.0 corresponded to liver fat content of 5.56%. There was no significant difference between men and women. Even though ours was not a population-based study, the prevalence of hepatic steatosis was similar in the current cohort as in the Caucasian American population in the DHS using ^{1}H-MRS (35% vs. 33%, respectively) (Browning *et al.*, 2004). Interestingly, in our cohort of healthy subjects who had undergone ^{1}H-MRS for measurement of liver fat content, the 95th percentile of liver fat was 5.9%, resembling the 5.56% found in the population-based DHS (Szczepaniak *et al.*, 2005). Our cohort was not population-based and thus the 5.56% in the DHS can be considered more accurate than our estimate of 5.9%.

The PNPLA3 I148M variant predisposes to NAFLD but not to features of MetS (Sookoian and Pirola, 2011). Thus, despite an increased liver fat content in PNPLA3 I148M variant carriers, HOMA-IR has been similar in carriers and non-carriers of similar age, gender and BMI, as shown in Table 5. Consistent with these data, in both of the present two healthy population-based cohorts, no difference existed in clinical characteristics between carriers and non-carries of the PNPLA3 I148M variant. The upper limit of normal HOMA-IR was the same in the carries and non-carriers. In the Liver Fat Cohort, in which 35% of subjects had NAFLD, the optimal cut-off for distinguishing NAFLD from non-NAFLD was also not affected by the genotype. However, if carriers are compared to non-carriers with a similar liver fat content, the carriers of the variant have significantly lower HOMA-IR than the non-carriers (III, Fig. 2b). These data suggests that HOMA-IR cannot be used for diagnosing subjects with 'PNPLA3 NAFLD', and that these subjects can only be identified by genotyping for this gene variant (EASL *et al.*, 2016).

6.4.2 Limitations in the use of HOMA-IR

As presented in the introduction, HOMA-IR is valid only as long as serum insulin concentrations reflect merely insulin sensitivity, not secretion (Bonora *et al.*, 2000; Borai, Livingstone, Farzal, *et al.*, 2010; Singh and Saxena, 2010). In subjects with non-diabetic glucose tolerance, fasting glucose and insulin concentrations have a close positive correlation (Lillioja *et al.*, 1988). Once glucose tolerance becomes diabetic, insulin concentrations start to decline and the relationship to glucose becomes inverse rather than positive (Lillioja *et al.*, 1988). Under such conditions, HOMA-IR underestimates insulin resistance associated NAFLD. As a fasting measure, HOMA-IR is also influenced by insulin clearance, unlike direct measurements of insulin sensitivity. However, this may not be a problem as the decrease in insulin clearance closely parallels that in hepatic insulin sensitivity (Kotronen, Vehkavaara, *et al.*, 2007).

Use of HOMA-IR in the clinic assumes that inter-laboratory variation in insulin assays is known (Manley *et al.*, 2008). In the present study, we analysed fasting blood samples, after a similar period of freezing, thawing and time of storage, from 10 individuals covering a wide range of HOMA-IR. Freezing and thawing the samples the same day had no significant impact on the values of fS-insulin but storage at -80°C degrees for 14 days caused a significant decrease in the values of fS-insulin. From the regression lines relating the assay results of two laboratories, the

upper limit of normal HOMA-IR was similar in Helsinki and Paris, where the same insulin assay was used (2.0), but 1.3 to 2.1 in the five other laboratories, which used different assays. The inter-laboratory CV was 25%, and this was due to the CV of fS-insulin (25.4%), not fP-glucose (5.4%). In contrast, the inter-laboratory CVs for other analytes, with the exception of ferritin, were much lower, ranging from 5% to 14%. This is in line with a previous study by the Insulin Standardization Workgroup, which found an inter-laboratory CV of 24% (Marcovina *et al.*, 2007).

The results of this study imply that every laboratory has to establish its own reference value for HOMA-IR or at least know how the insulin assay compares with that of other laboratories. Furthermore, reference values for HOMA-IR, even of subjects defined as healthy, and the relationship between HOMA-IR and liver fat may be population-specific.

6.4.3 Performance of fS-pIGFBP-1 as a non-invasive predictor of liver fat content in NAFLD

In study IV, it was shown that measurement of fS-pIGFBP-1 might help in the prediction of liver fat content in the face of other correlates of liver fat. The final model for predicting liver fat included age, fS-pIGFBP-1, an interaction term (age x fS-pIGFBP-1), S-ALT, waist-to-hip ratio, fP-glucose and fS-insulin. This study is the first to measure pIGFBP-1 rather than IGFBP-1 and in combination with other known predictors of liver fat content.

In the previous studies measuring IGFBP-1, the extent to which the assay measured phosphorylated forms of IGFBP-1 was not specified (Alderete *et al.*, 2011; Maddux *et al.*, 2006; Rajpathak *et al.*, 2009; Savastano *et al.*, 2011; Wasada *et al.*, 2008). In the present study, the median fS-pIGFBP-1 was 58 µg/l, which is similar to the earlier reported pIGFBP-1 concentrations ranging from 29 to 100 µg/l (Borai, Livingstone, Ghayour-Mobarhan, *et al.*, 2010; Coverley and Baxter, 1997; Heald *et al.*, 2002). These concentrations are notably higher than those of lesser-phosphorylated IGFPB-1 ranging from 4 to 12 µg/l (Borai, Livingstone, Ghayour-Mobarhan, *et al.*, 2010; Heald *et al.*, 2002). The phosphorylation status of IGFBP-1 alters its antigenicity (Jones *et al.*, 1991) and consequently some immunoassays may grossly underestimate changes in IGFBP-1 concentrations (Mehta *et al.*, 2012). Previous RIAs used for measuring IGFBP-1 produced mean fS-IGFBP-1 concentrations ranging from 16 to 20 µg/l (Kotronen, Lewitt, *et al.*, 2008; Lewitt *et al.*, 2010; Mohamed-Ali *et al.*, 1999) and detected thus only a fraction of total IGFBP-1. In line with this, in the present study, both IGFBP-1 using RIA and pIGFBP-1 using IEMA were measured in the subset of 23 subjects. The mean concentration of fS-IGFBP-1 using RIA (18 µg/l) was much lower than that of fS-pIGFBP-1 using IEMA (58 µg/l).

The inverse relationship between liver fat content and pIGFBP-1 is in line with earlier data in diverse groups measuring IGFBP-1 using RIA or an immuno-radiometric assay (Alderete *et al.*, 2011; Kotronen, Lewitt, *et al.*, 2008; Savastano *et al.*, 2011; Wasada *et al.*, 2008). In the studies by Savastano et al. in 48 subjects (Savastano *et al.*, 2011) and Kotronen et al. in 113 subjects (Kotronen, Lewitt, *et al.*, 2008), the correlation coefficients between fS-IGFBP-1 and hepatic steatosis score (US) or liver fat (^{1}H-MRS) were in both studies -0.38 ($p<0.01$ or less). In the present study, the correlation coefficient between fS-pIGFBP-1 and liver fat (^{1}H-MRS) in 378 subjects was -0.27 ($p<0.0001$). This was statistically comparable to the previous data in smaller groups of subjects ($p=0.5$ for $r=-0.27$ in 378 subjects vs. $r=-0.38$ in 48 subjects (Savastano *et al.*, 2011) and $p=0.3$ for $r=-0.27$ in 378 subjects vs. $r=-0.38$ in 113

subjects (Kotronen, Lewitt, *et al.*, 2008)).

As discussed, causes (aging, body composition) and consequences (hyperinsulinemia, hypertri-glyceridemia, hyperglycaemia, elevated liver enzymes) of insulin resistance are known to be significantly associated with increased liver fat. Therefore, several variables rather than one alone should be examined when developing tools for non-invasive prediction of liver fat. In addition to the previously established markers, fS-pIGFBP-1 was significantly associated with liver fat content in both univariate and multiple linear analyses. The PNPLA3 I148M variant significantly associated with liver fat in univariate but not in multiple linear regression analyses. This might be due to the fact that the variant could signal its influence via increased S-ALT, which remained a significant independent predictor in multiple linear regression analysis. Subjects with increased liver fat content were also hyperinsulinemic, possibly contributing to the observed inverse relationship between fS-Insulin and fS-pIGFBP-1. Consequently, the relationship could reflect inhibition of production of IGFBP-1 in the liver by insulin (Brismar *et al.*, 1994). Hepatic insulin resistance could also influence the slope of the relationship between fS-insulin and fS-IGFBP-1. A fixed increment in serum insulin suppresses serum IGFBP-1 less in subjects who are insulin-resistant than in the insulin-sensitive (Kotronen, Lewitt, *et al.*, 2008). Of these two factors, i.e., insulin per se and hepatic insulin sensitivity, insulin might be more important in regulation of fS-IGFBP-1, as type 1 diabetic patients who lack the portal-peripheral insulin gradient have markedly higher fS-IGFBP-1 concentrations than matched non-diabetic subjects, even in cases where the latter have enhanced hepatic insulin sensitivity (Llauradó *et al.*, 2015; Yki-Järvinen *et al.*, 1995).

In line with the observed significant interaction between age and fS-pIGFBP-1, IGFBP-1 correlates with age independent of BMI (Rutanen *et al.*, 1993). Aging associates with decreased suppression of IGFBP-1 by insulin (Rutanen *et al.*, 1993). The variables that are used in equations to evaluate liver fat should be standardised in order to enable comparison between different laboratories and centres. Although fS-insulin is perhaps the most popular laboratory test for assessing insulin sensitivity, its assay procedures, as discussed earlier and shown in this study, are highly variable and have high a inter-laboratory variation. The measurement of pIGFBP-1 for predicting pre-term delivery (Rahkonen *et al.*, 2009; Riboni *et al.*, 2011) and of lesser-phosphorylated IGFBP-1 for the diagnosis of premature rupture of foetal membranes (Rutanen *et al.*, 1996) have become worldwide standards and are produced by a single manufacturer.

Limitations in the study are acknowledged. The study was cross-sectional and thus cause and consequence cannot be proven. Also, even when a great number of factors known to be either causes or consequences of liver fat content were examined, a large proportion of the variation in the liver fat is unexplained. Direct measurement of liver fat content by widely available US would seem to be a more attractive tool. As discussed earlier, limitations of the use of US is that that the sensitivity is poor in subjects with low liver fat content (Ryan *et al.*, 2002) and accuracy is weak in obese subjects (Mottin *et al.*, 2004). Compared to measurement of e.g. liver enzymes alone, the '%Liver Fat Equation' was much better in capturing information on liver fat. The AUROC of the '% Liver Fat Equation' (0.84) was significantly better than that of the FLI (0.72) or the HSI (0.62). The '% Liver Fat Equation' has been published as an online supplement and is available for calculation at www.nature.com/articles/srep24720 (Petäjä *et al.*, 2016).

7 SUMMARY AND CONCLUSIONS

The present studies were undertaken to gain further knowledge of the pathogenesis and diagnosis of NAFLD.

Pathogenesis
Regarding pathogenesis, we found abdominal SC adipocyte size to be associated with liver fat independent of other known associates, including obesity, adipose tissue distribution, *PNPLA3* genotype and age. This helps to explain why adipocyte hypertrophy has been associated with features of insulin resistance and of the metabolic syndrome independent of obesity and fat distribution. In addition, these data may also help to explain why both adipocyte hypertrophy and liver fat predict type 2 diabetes independent of age, gender and obesity.

Diagnosis
Definitions of normal liver fat vary depending upon the diagnostic method used and do not correspond directly with each other. This needs to be considered when guidelines for diagnosis of NAFLD are established (II).

NAFLD is heterogeneous. Insulin resistance characterises 'Metabolic NAFLD' and can be estimated using HOMA-IR. We found in two population-based cohorts that the upper reference limit of HOMA-IR in healthy subjects is 1.9-2.0. These values were not affected by gender or genetic variation in *PNPLA3*. In ROC-analysis, a HOMA-IR of 1.9 was the best for distinguishing between NAFLD and non-NAFLD. In linear regression analysis, normal liver fat content corresponded to a HOMA-IR of 2.0 with no differences between men and women. A HOMA-IR exceeding 2.0 in our laboratory thus suggests the patient has NAFLD. However, inter-laboratory variation in insulin assays and thus in HOMA-IR is high (III). In contrast to 'Metabolic NAFLD', no difference in insulin sensitivity was observed in 14 studies between carriers and non-carriers of the PNPLA3 I148M variant. Similarly, no difference in insulin sensitivity was observed in 7 studies comparing insulin sensitivity between carriers and non-carriers of the TM6SF2 E167K variant (II). Carriers of the PNPLA3 I148M variant had significantly lower HOMA-IR in the face of similar liver fat content than the non-carriers. Use of HOMA-IR to diagnose NAFLD in carriers of these gene variants thus underestimates liver fat content.

pIGFBP-1 helps in the prediction of liver fat content in NAFLD compared to routinely available clinical and biochemical parameters (IV). Its measurement may be helpful in diagnosing NAFLD.

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